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INDEX

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Prenylamine and Brain Monoamine Metabolism

By

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In recent years a good deal of interest has been paid to prenylamine (segontin ®), used clinically as a coronary dilator The compound shows many striking resemblances to reserpine. Thus it reduces the concentrations of the catecholamines noradrenaline (NA) and dopamine (DA) (Schöne & Lindner 1960 Juorio & Vogt 1965 Obianwu 1965) in the rat brain, though the depleting effect on brain 5-hydroxytryptamine (5-HT) is reported to be less marked (Schöne & Lindner 1960 Obianwu 1965), or lacking (Juorio & Vogt 1965 Fresia *et al* 1963) The amine depleting effect is also seen in various other tissues, *e.g* the heart (see also Mackenna 1965)

In addition to the prolonged monoamine depletion in the brain, reserpine also causes an accumulation of various acid monoamine metabolites, formed after the action of amine metabolizing enzymes. Thus the levels of 3 4-dihydroxyphenylacetic acid (DOPAC) (Andén *et al* 1963a), homovanillic acid (HVA) (Sharman 1963 Andén *et al* 1964), and 5-hydroxyindoleacetic acid (5-HIAA) (Ashcroft & Sharman 1962 Roos & Werdinius 1962) are all raised in the brain after reserpine, the two latter acids still showing increased concentrations after twentyfour hours.

The work reported here was undertaken to see whether prenylamine had effects similar to reserpine on the levels of the acid monoamine metabolites mentioned above.

Experimental Methods

Prenylamine lactate (5 mg/kg, calculated as the base) was given in single slow intravenous injections to adult rabbits (1 1 2.5 kg) of both sexes, usually three animals in each experiment. The animals were then killed by air embolism at various intervals. In order to

minimize any possibl diurnal variations, the prenylamine injections were timed so as to allow analyses t be made at bout the same time each morning. The animals were kept at an environmental temperature of 26° during the treatment. In some cases the body temperature was checked throughout the experiments. At regular intervals throughout the experimental period untreated animals were used for the determination of normal values.

Brain dissections. As soon as possible after sacrifice the brains were removed and dissected. The corpora striata (nucleus caudatus and nucleus lentiformis) from three brains were pooled and analysed for DOPAC and HVA. The weight of these brain tissues ranged between 0.31 and 0.53 g per pair For each DA estimation one brain was dissected in the same way and nalysed separately The remainder of the brains was freed from cortical and cerebellar tissue, and the portions anterior to the caudal colliculi, mainly consisting of the thalamus, hypothalamus and mesencephalon were pooled and taken for analysis of 5-HIAA and 5-HT Each "anterior brain stem" preparation so obtained weighed between 1.26 and 1 71 g per animal.

Estimation of DOPAC and HVA The tissue was hom genized in 60 ml ice cold 10 % (w/v) metaphosphoric acid. The further extraction procedure and fluorometric analysis were essentially performed as briefly outlined previously (Andén *et al.* 1963 a, b) but will be given here in detail. To one half of the homogenat known amounts of DOPAC and HVA (4–6 µg) were dded to check the recovery and the two portions were then treated in the same way throughout the procedure. The centrifuged and filtered extract was washed at neutral pH with 300 ml heptane. The aqueous phase (about 30 ml) was re-acidified with 3 ml 5 N HCl, saturated with sodium chloride, and extracted with 300 ml ether The ether phase was reduced to 50 ml (water bath 30° rotating evaporator reduced pressure), kept at −70° for on hour (solid carbon dioxide in ethanol) in order t freeze out water with remaining impurities, and rapidly filtered while still cold. The DOPAC and HVA were then re-extracted with 6 ml "tris buffer (trishydroxymethyl-aminomethane 0.05 M, pH 8.5).

DOPAC was determined fluorometrically by condensation with ethylene diamine (Kägi et l 1957 Andén et al. 1963). To 1 0 ml of the tris buffer sample was added 0.4 ml ethylene diamine reagent (ethylene diamine 4 M ammonium chloride 1 13), and the mixture kept at +63 for 20 min. The resulting fluorescence was read in an Aminco-Bowman spectrophotofluorometer with the activating and fluorescent wavelengths set at 415 and 540 mµ, respectively (uncorrected instrumental values). The activation and fluorescent spectra of the sample and a standard of authentic DOPAC were in close greement. Tissue blanks were obtained by running piece f cerebella tissue f th same weight throughout the analysis (Andén et al. 1963)

HVA was assayed fluorometrically in the remaining tris buffer sampl by the xidation procedure of Andén et l. (1963 b) To 1 0 ml sample were added, in the following order 0.2 ml water 1.0 ml 5 N mmonia, and 0.2 ml 0.01 % potassium ferricyanide, f llowed after four min. by 0.2 ml 0.1 % L-cysteine. The fluorescence was read at 310 mµ activating and 425 mµ fl orescent wavelengths (uncorrected values). An internal standard (with 2 µg HVA added to the buffer instead of water) was treated in parallel with the sample and a standard of authentic HVA in order to check the oxidatio yield (ranging between 95 and 105 %). A tissue blank was btained by mixing the xidant and the cysteine before adding the buffer sample and ammonia, thus preventing the oxidation.

The recovery percentages throughout the procedure varied in the range 74.2 ± 13.2 for DOPAC, and 79 9 ± 7.2 for HVA (mean ± s.d.) when due aliquot corrections were made (except for the loss of ether during filtration, about 10 %). The recoveries are in reasonably good agreement with those expected theoretically from the distribution coefficients of the substances between the organic and aqueous phases. The results were corrected for the individual recoveries in each experiment.

Estimation of 5-HIAA and 5-HT The pooled anterior brain stem preparations were homogenized in 60 ml ice cold 0.1 N-HCl with the ddition of 50 mg ascorbic acid. After dding known amounts f 5-HIAA and 5-HT (?–3 μg) to one half of the homogenate, the two portions were treated in the same way throughout the procedure. The proteins were precipitated with zinc hydroxide (10 ml 10 % $ZnSO_4 \cdot 7H_2O$ and 5 ml 1 N NaOH) The further extraction procedure for 5-HIAA was identical with th t described bove for DOPAC and HVA except that the tris buffer was replaced by 3.5 ml 0.25 M phosphate buffer pH 7.0.

The 5-HT still remaining in the acid aqueous phase after the ether extraction, was then extracted according to BOGDANSKI *et al.* (1956) with slight modifications. The salt saturated aqueous phase (bout 35 ml) was made alkaline with solid sodium carbonate, and 20 ml 0.5 M borate buffer pH 10 (saturated with butanol and sodium chloride) was added. Th 5-HT was extracted into 40 ml butanol, which was then washed with an equal volume of borate buffer To 30 ml of the butanol 60 ml heptane were added, and the 5-HT was subsequently re-extracted int 3 ml 0.5 N HCl.

I the final extracts both 5-HIAA and 5-HT were assayed by their fluorescence in 3 N-HCl, according to the method originally described for 5-HT by UDENFRIEND *et al.* (1955). Some modifications (ANDÉN & MAGNUSSON 1967) allowed tissue blank t be read at the same time. The fluorescence was read at 295 mμ activating and 545 mμ fluorescent light (uncorrected values), with UV filter fixed in the filter holder in front of the photo cell. This eliminates the light scatter which would otherwise interfere with the readings.

The recoveries throughout the procedure were in the range 61 6 ± 9.2 for 5-HIAA, and 44 4 ± 7.3 for 5-HT (mean ± s d.). Experiments with pure standards of different concentrations gave recoveries in very close agreement with these figures, indicating that factors like protein binding did not affect the recoveries to any great extent. All results were corrected for the individual recoveries in each experiment.

I a few additional experiments, 5-HT was determined by the method of BERTLER (1961), involving separation by ion-exchange chromatography before fluorometric estimation. The results agreed reasonably well with those obtained by the present method (70–100 % of the means at the various time intervals).

Estimation of DA was carried out by the method of CARLSSON & WALDECK (1958), with the modifications described by CARLSSON & LINDQVIST (1962).

Results

Normal values of DOPAC, HVA, DA, 5-HIAA and 5-HT in the different parts of the rabbit brain mentioned previously are given in table 1 which also summarizes the effects produced by prenylamine, 5 mg/kg intravenously

Three hours after the injection of prenylamine, there was a moderate increase in the brain stem 5-HIAA ($P < 0.05$), approximately correspond ing to a moderate decrease in the brain stem level of 5-HT ($P < 0.05$) In the corpus striatum, the levels of the DA metabolites DOPAC and HVA were significantly increased at three hours, to about 250 and 150 % respectively of the normal value ($P < 0.025$ and $P < 0.0005$) The DOPAC values, however varied rather widely in the different expe-

Table 1

DOPAC HVA, DA, 5-HIAA and 5-HT in rabbit brain at various intervals after intra venous administration of a single dose of prenylamine 5 mg/kg.

The values (corrected for recovery) are given in µg/g tissue ± s.e.m. Numbers of analyses are given in brackets, and each single experiment was made on three pooled brains (for DA one brain).

Time interval hours	Corpus striatum			Anterior brain stem	
	DOPAC	HVA	DA	5-HIAA	5-HT
C trol	0.71 ± 0.12 (6)	3.24 ± 0.24 (8)	3.47 ± 0.13 (2)	0.96 ± 0.11 (7)	0.77 ± 0.11 (5)
1	1.32 ± 0.18 (2)	5.21 ± 1.[illegible]3 (3)	1.05 (1)	1.06 ± 0 08 (2)	0 49 ± 0.03 (3)
3	1 73 ± 0.43 (5)	5 17 ± 0.37 (6)	0.85 ± 0.12 (7)	1.25 ± 0 11 (7)	0.50 ± 0.05 (5)
	P < 0.025	P < 0.0005		P < 0 05	P < 0.05
6	0.51 (1)	3 79 ± 1 17 (2)	1.57 (1)	1.[illegible]4 ± 0.14 (2)	0.43 (1)
1[illegible]	0.48 ± 0.17 (3)	3.09 ± 0.12 (3)	3.41 ± 0.08 (2)	1 10 ± 0.17 (2)	0.75 ± 0.02 (3)

riments. Simultaneously the DA level was reduced to about 25 / of the normal value.

The same tendency with regard to amine and metabolite levels also occurred at one hour after the prenylamine injection but to a slighter extent or with greater fluctuations The levels seemed to have returned to normal within twelve hours.

Up to half an hour after the injection of prenylamine the animals showed signs of agitation, with mydriasis, nystagmus, tremor and ataxia. Occasionally moderate, short-lasting, intermittent convulsions occurred shortly after the injection. One or two hours later the animals were slightly sedated but they showed no other signs typical of reserpine treatment, such as miosis or diarrhoea. Higher doses than 5 mg/kg were tried but had to be abandoned because of convulsions, sometimes terminating in death

The body temperature was normal (around 38.5°) throughout the treat ment, except for the first 30 min following the injection, when increased muscular activity led to a moderate increase in temperature (0.5–0 8°)

Discussion

The experiments reported here showed that, in the rabbit brain, prenylamine significantly increased the DOPAC and the HVA content of the corpus striatum and, in parallel, lowered the DA in the corpus stri atum. Moreover it produced some increase in the brain stem 5-HIAA and a corresponding lowering of the brain stem 5-HT The maximal

effects seemed to occur at three hours At twelve hours the levels appeared to have returned to normal.

Prenylamine thus seems to bring about the same metabolic effects on the brain monoamines as reserpine, although to a much smaller extent. As has been shown earlier reserpine produces within two hours a two- to threefold increase of DOPAC and, somewhat later of the HVA levels in the rabbit corpus striatum, in parallel with the prolonged dopamine depletion (ANDÉN *et al* 1963 a & 1964 ROOS *et al* 1964 SHARMAN 1963). Similarly the 5-HIAA concentration of the brain stem is approximately doubled within two hours, while 5-HT is reduced to insignificant levels after reserpine, the metabolite increase being approximately stoichometrichal to the amine depletion (ROOS & WERDINIUS 1962) The DOPAC level falls to normal in twelve hours, whereas the increased HVA and 5-HIAA levels still persist after twenty-four hours. These data lend further support to the view that the amines 5-HT and DA, when released from the intra neuronal storage sites into the cytoplasma by reserpine, are rapidly converted by the mitochondrial enzyme monoamine oxidase to the acid metabolites 5-HIAA and DOPAC respectively These products disappear from the brain only slowly and the DOPAC is gradually converted to HVA by the enzyme catechol-O-methyl-transferase, probably localized outside the amine producing cell (cf CARLSSON 1960 CARLSSON & HILLARP 1962).

JUORIO & VOGT (1965) found a decrease of brain stem DA and hypothalamic noradrenaline in rats after subcutaneous administration of prenylamine. These effects lasted at least twenty four hours. In contrast, no significant effect was noted on the brain content of 5-HT whereas a moderate increase occurred with 5-HIAA, lasting for at least twenty-four hours. Brain stem HVA did not show any significant deviations from the very low basal value found in rats (cf JUORIO *et al.* 1966) There was also no change in the HVA in the caudate nucleus of rabbits, although a marked decrease occurred in the DA content four hours after a subcutaneous injection of prenylamine 100 mg/kg. The partial discrepancy in results might be due to species differences, dose, and delayed absorption from the site of injection.

The effects of reserpine on both brain monoamine metabolism and general behaviour are more marked and prolonged than those of prenylamine, which caused only slight sedation, coincident with the maximal effects on brain monoamine metabolism. CARLSSON & LINDQVIST (1966) have shown that pretreatment of mice with prenylamine shortly before reserpine administration partially protected the animals against the longlasting actions of reserpine on behaviour and on brain monoamine levels. This might indicate that the two drugs compete for the same

sites of action. Several experiments on isolated amine containing granules further support the similarity with reserpine. Thus, the specific ATP Mg^{++} dependent mechanism for incorporation of catecholamines into the storage granules of the adrenal medulla and peripheral adrenergic nerves is selectively blocked by reserpine as well as by prenylamine in low concentrations (CARLSSON *et al.* 1963, EULER & LISHAJKO 1965, LUNDBORG 1966). On the other hand, none of the substances seem to block the amine transport mechanism of the cell membrane (CARLSSON & WALDECK 1965).

The sedative action of prenylamine is weak, as mentioned above, and the monoamine depleting action moderate. Nevertheless, after treatment with prenylamine for five days in man, certain reserpine-like actions have been reported, such as bradycardia, and reduced response to the pressor action of tyramine (KUSCHKE *et al.* 1964, 1965).

Summary

A single dose of prenylamine (segontin ®), 5 mg/kg given intravenously to rabbits, significantly increased the concentrations of the acid dopamine metabolites 3,4-dihydroxyphenylacetic acid and homovanillic acid in the corpus striatum, in parallel with a decrease in the dopamine content. In the brain stem, the level of 5-hydroxyindoleacetic acid was moderately increased, corresponding to a moderate decrease in 5-hydroxytryptamine. Maximal effects on the amine metabolism were noted about three hours after the prenylamine administration, and the changes indicate an increased monoamine degradation. These results are in line with the view that prenylamine has the same mode of action as reserpine, though much weaker and of shorter duration.

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Elimination of 3,4-Dihydroxyphenylacetic Acid from the Blood

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(Received August 10, 1966)

One major metabolite of dopamine, namely 3 4-dihydroxyphenylacetic acid (DOPAC), is formed after oxidative deamination by the enzyme monoamine oxidase (MAO) and subsequent oxidation of the intermediary aldehyde by aldehyde dehydrogenase (LEEPER, WEISSBACH & UDENFRIEND 1958) DOPAC occurs normally in the central nervous system (EULER 1958), mainly in the dopamine containing structure corpus striatum, where the concentration is about 0 5–0 7 µg/g in the rabbit (ROSENGREN 1960 ANDÉN ROOS & WERDINIUS 1963a) It is, at least partly metabolized by the enzyme catechol-O-methyltransferase (COMT) to homovanillic acid (HVA) The presence of HVA in the brain, particularly in the corpus striatum, has been demonstrated in a number of species (SHARMAN 1963 ANDÉN, ROOS & WERDINIUS 1963b BERNHEIMER 1964 JUORIO & VOGT 1965 JUORIO, SHARMAN & TRAJKOV 1966)

In addition to the brain, DOPAC has also been shown to occur normally in small amounts in bovine blood plasma (EULER, FLODING & LISHAJKO 1959) The concentrations detected were about 12–40 µg/l, the amounts increasing somewhat after acid hydrolysis.

Both DOPAC and HVA also occur normally in urine (EULER, EULER & FLODING 1955 EULER, FLODING & LISHAJKO 1959 ARMSTRONG SHAW & WALL 1956). The urinary DOPAC is probably at least partly derived from the DOPAC in the blood.

It was considered of interest to study the elimination pattern of exogenously administered DOPAC from the blood.

Materials and Methods

Rabbits (1 –1.9 kg) of either sex were generally used for the experiments. DOPAC (3,4-dihydroxyphenylacetic acid Fluka) was administered intravenously (50, 100 or 500 mg/kg) In some experiments the animals were pretreated with various drugs, one hour before the DOPAC injection These drugs include the COMT-inhibitor 4-tropolone-acetamide (H 17/27), 100 mg/kg intravenously probenecid 100 mg/kg, and diethylamino-ethyl diphenylpropylacetate (SKF 525A), 10 mg/kg. All the compounds were dissolved in saline and neutralized to pH about 7–7.5 Samples of venous blood (bout one ml) were drawn from the uninjected ear regularly during the following 2–3 hours at 15 to 30 min. intervals, the first sample just before the DOPAC injection. The blood was collected in test tubes containing 0.1–0.2 mg (10–20 IE) dry heparin. The blood samples were stored in the refrigerator until analyzed, usually for not more than few hours. No decrease was, however observed if the analysis was repeated on the next day

Assay of DOPAC in blood or plasma DOPAC was determined fluorimetrically by condensation with ethylene diamine (NATELSON, LUGOVOY & PINCUS 1949 KÄGI, BURGER & GIGER 1957 WEIL MALHERBE 1959). To 2.5 ml saline in centrifuge tubes 0.2 ml blood was added, and the corpuscles were spun down. Two ml of this supernatant were transferred to new centrifuge tubes, and the plasma proteins precipitated with 0.5 ml 10 % (w/v) meta phosphoric acid. After centrifugation, one ml of the supernatant was transferred to glass-stoppered test tubes, and 0.4 ml of freshly prepared ethylene diamine reagent (ethylene diamine and 4 N ammonium chloride in the proportions 1 1.3) was added. The tubes were heated to 63 for 20 min. and then cooled. The fluorescence was read in an Aminco-Bowman spectrophotofluorometer at 415 mμ activating and 540 mμ fluorescent wave-lengths (uncorrected instrumental values), gainst standard of uthentic DOPAC. Activation and fluorescence spectra agreed closely with those of authentic DOPAC. The recovery ranged between 88 and 104 %, as checked by internal standards carried through the entire procedure. N loss of DOPAC occurred during the protein precipitation This was further confirmed by re-extraction experiments. The concentration of catechol compounds forming fluorescent condensation products with ethylen diamine is normally very low in blood and plasma hence, the zero time blood sample could serve as a blank.

The DOPAC analyses in plasma were performed essentially as described for blood, but omitting the first step.

If the red corpuscles were hemolyzed in the whole blood procedure (saline exchanged f water in the procedure), there was some interference with the final fluorescence, giving considerably lower reading values. Essentially the same results were btained after correc tio for the low recovery indicating either that no DOPAC was present in the erythrocytes, or that the DOPAC content f the erythrocytes rapidly re-equilibrated with the saline-diluted plasma in the first step of the procedure. In fact, the DOPAC content of the whole blood seemed to be almost exclusively confined to the plasma (cf CARLSSON & HILLARP 1962), as evidenced by simultaneous determinations on blood and plasma, and assay of the hematocrit value (generally 28–36 %) For technical reasons most f the analyses were performed on whole blood.

Assay of HVA in blood 0.2–0.5 ml f the blood was diluted to 3 ml with saline. The procedure was then the same as described bove for DOPAC, p to the protein precipation. Two ml of the protein-free supernata t were transferred to glass-stoppered extraction tubes, acidified with 0.5 ml 6 N HCl and extracted with 50 ml ether f 15 min. The HVA was then re-extracted from the ether into 4 ml "tris buffer (trishydroxymethyl-amino-methane 0.05 M, pH 8.5) and assayed according to the oxidation procedure of ANDÉN, ROOS & WERDINIUS (1963 b). Appropriate corrections were made for recovery (65 to 85 %, as checked by internal standards).

Chromatographic examination of blood. The blood (0.5–0.8 ml) was extracted as described bove for HVA, except that the acidified aqueous phase was saturated with sodium chloride during the ether extraction. The ether was then evaporated to dryness (rotating evaporator reduced pressure), and the residue dissolved in a small volume f ethanol which was then applied to paper (Munktell S 302) and subjected to ascending chromatography The solvents used were butanol-acetic acid-water 4 1 1 or butanol-pyridine water 14 4 5 The chromatograms were developed with diazotized *p*-nitroaniline.

Results and Discussion

The contents of DOPAC and HVA were too low in the blood from normal rabbits (less than 0.2 μg/ml) to allow accurate determinations with the methods used After administration of DOPAC (50 or 100 mg/kg intravenously), however large amounts of DOPAC were demonstrated in the blood during the next hour It was rapidly eliminated from the blood stream, and generally after 2–3 hours no significant amounts of DOPAC could be detected. The HVA level began to rise immediately reaching its peak value at 30 to 90 min Even after 3 hours, significant amounts of HVA could generally be demonstrated. Paper chromatograms of blood samples revealed only two spots corresponding to DOPAC and HVA, respectively

When the blood (or plasma) concentrations of DOPAC were plotted in a semilogarithmic diagram (log concentration against time), a linear relationship was apparent (fig. 1) This indicates that the overall elimination of DOPAC was of the first order *i.e* the elimination rate is proportional to the remaining blood concentration of DOPAC.

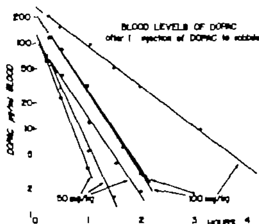


Fig. 1 Blood levels of DOPAC at various time intervals fter the intravenous administration of DOPAC (50 or 100 mg/kg) into different rabbits.

2*

Three elimination routes for DOPAC seem to need consideration 1) Excretion of DOPAC by the kidneys, 2) conversion of DOPAC to HVA, which is eventually excreted, and 3) conjugation of DOPAC with subsequent excretion of the conjugate. From the linear semilogarithmic plots of the overall elimination from blood, it is evident that the main elimination routes at least are of the first order whereas minor routes might possibly be saturated at higher blood DOPAC concentrations, *i.e.* be of the zero order reaction

In order to evaluate the relative importance of the different routes of renal elimination, the urine was collected at 30 min intervals from a rabbit which had been given DOPAC 50 mg/kg intravenously The rabbit was anaesthetized (20 / urethan, 4 ml/kg i v) and catheters were inserted into the ureters for the collection of urine. Blood analyses showed no change in the elimination rate of DOPAC as compared with that in the unanaesthetized animals therefore, the effect of the anaesthesia appeared to be unimportant. After two hours, 60 / of the administered DOPAC had been eliminated unchanged, while only 0.3 / was excreted as HVA. After five hours, the corresponding figures were 73 / and 2.5 /, respectively The elimination was not yet complete at this time, as considerable amounts of both DOPAC and HVA were demonstrable in the last urine portion. Acid hydrolysis of the urine (N-HCl 1 hour) did not increase the values significantly Paper chromatography of the urines (2 µl of urine applied directly on the paper) revealed two spots, one large and one

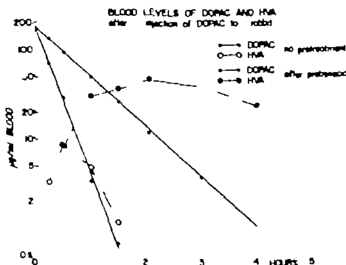


Fig — Blood levels f DOPAC a HVA at various time intervals fter dministration of DOPAC (50 mg/kg) into rabbit. The effect of probenecid pretreatment (100 mg/kg 1 hour) is shown i the same rabbit.

Table 1

Half life ($t_{½}$), apparent initial concentration in blood (C_0), and coefficient of distribution with regard to blood (k), in rabbits after the i.v. administration of DOPAC. Various pretreatments (H 17/27 100 mg/kg; probenecid 100 mg/kg; SKF 525A 10 mg/kg) were given intravenously one hour before the DOPAC injection.

Rabbit no.	DOPAC mg/kg	Pretreatment	$t_{½}$ min	C_0 µg/ml	k ml/g
1	50	–	14	120	0.42
2	50	–	11	190	0.26
	50	Probenecid	32	180	0.28
	50	H 17/27	11	190	0.[illegible]6
	50	H 17/27+probenecid	27	190	0.[illegible]6
3	50	–	17	160	0.31
	50	SKF 525A	15	160	0.31
	50	Probenecid	44	175	0.29
4	50	–	11	140	0.36
5	50	–	12	115	0.43
	50	–	10	115	0.43
6	50	–	9	130	0.38
7	100	–	20	240	0.42
	100	H 17/27	19	240	0.4[illegible]
	100	–	19	240	0.42
8	100	–	39	250	0.40
9	500	H 17/27	22	1650	0.30

smaller corresponding to DOPAC and HVA respectively. In the earlier samples of urine, two very small additional spots were noted near the origin, which might possibly represent conjugates. The main route for the elimination of exogenous DOPAC thus seems to be renal excretion of unchanged DOPAC.

The half-life of DOPAC in the blood was easily evaluated from the semilogarithmic plots (see fig. 1 and 2), obtained by drawing the best-fitting line through the points. Considerable variation was noted between different animals, whereas the elimination rate was rather constant in the same animal in different experiments. The half-life of DOPAC ranged between 9 min. and 39 min. (mean 18 min., 8 animals) see table 1.

These large variations may, in part, be attributed to certain strain factors. The acid-base state of the animal may also be important. At a relatively low urinary pH the renal tubular reabsorption of DOPAC might be considerable, thus causing an increase in the half life, whereas a higher urinary pH might promote the excretion and thus reduce the half-life. The well known sensitivity of salicylic acid excretion to urinary

pH is noteworthy in this connection (WILLIAMS & LEONARDS 1948 SMITH 1949 LEVY 1965)

The rapid urinary excretion of DOPAC could not possibly be explained, unless active renal secretion mechanisms were involved. As is well known, probenecid decreases the tubular secretion of a variety of organic acids. In some of the experiments the animals were therefore pretreated with probenecid 100 mg/kg, one hour before the administration of DOPAC (50 mg/kg) The results of a typical experiment are given in fig. 2, together with an experiment in the same rabbit without probenecid pretreatment. The elimination of DOPAC was still of the first order but the half life increased 2.5–3 times in the probenecid pretreated animals (see table 1) The HVA levels also rose much more in the pretreated animals, and the time course of elimination was markedly prolonged. In other experiments HVA (50 mg/kg i.v.) was injected, and the elimination was found to be of the first order After probenecid pretreatment (100 mg/kg i.v.), the half-life of HVA increased from 25 min. to 64 min. There is thus good support for the view that both DOPAC and HVA are eliminated by an active renal process, which is inhibited by probenecid. DESPOPOULOS & WEISSBACH (1957) have demonstrated a similar mechanism in man for the main metabolite of serotonin, 5-hydroxyindoleacetic acid the urinary elimination of which is partly blocked by probenecid

Even at very high doses of DOPAC (500 mg/kg i.v.), there was no demonstrable saturation effect on the elimination of DOPAC, which still proceeded by apparently strict first order kinetics (half-life 22 min.).

In attempts to block other possible elimination routes for DOPAC, the COMT inhibitor H 17/27 (4-tropolone-acetamide see CARLSSON, CORRODI & WALDECK 1963) was administered (100 mg/kg i.v.) one hour before the DOPAC injection. The COMT appeared to be completely blocked, since no HVA could be demonstrated either in blood or in urine. In spite of this, the DOPAC elimination proceeded at exactly the same rate as in control experiments in the same animals without pretreatment. In other experiments, the animals were pretreated with SKF 525A (diethylaminoethyl diphenylpropylacetate, 10 mg/kg i.v.) no effect was, however observed This substance seems to inhibit a number of drug transforming enzymatic reactions in the microsomes, including certain types of conjugation (see NETTER 1962)

The apparent initial concentration of DOPAC in the blood, which would have been present if the distribution equilibrium had been immediately achieved, is obtained by extending the linear semilogarithmic plots to zero time This concentration, C_0 (μg/ml), is approximately proportional to the dose administered, D (mg/kg or μg/g), *i.e.* k $C_0 = D$ (see DOST 1953 SWINTOSKY 1956) The factor of proportionality k, with

the dimension ml/g, is the coefficient of distribution with regard to blood This factor has often been called the apparent volume of distribution (DOST 1953). It must not be interpreted as an actual volume, unless the substance is not bound. Binding to the plasma proteins would decrease the k value, provided that the assay procedure includes the total concentration of the substance (free and protein bound) in the blood or plasma. Binding to extravascular sites (e g the active neuronal uptake of catecholamines from the circulating blood) would on the other hand increase the apparent volume of distribution.

The C_0 and k values for DOPAC in the different experiments are given in table 1 A certain variation in the k value is apparent between the different animals, whereas the fluctuation of k between the different experiments in the same animal seems to be considerably less. The reasons for this are obscure, but individual differences in body composition, such as the relative amount of fat depots, could possibly be involved.

If the mean k value (0.38 ml/g) was corrected for the mean hematocrit value (33 %) the resulting coefficient of distribution with regard to *plasma* would be 0.25 ml/g. This correction seems to be justified, since DOPAC penetrates into the erythrocytes only to a slight extent (see above) The value might imply that DOPAC was mainly distributed to the extra cellular fluid. However until studies on the binding of DOPAC to plasma proteins have been performed, no definite conclusions can be drawn.

Summary

When 3 4-dihydroxyphenylacetic acid was administered intravenously to rabbits in various doses (50 to 500 mg/kg), the elimination from the blood proceeded rapidly by apparently first order kinetics (half-life 9 to 39 min.) Even at the highest dose used, no saturation effect on the elimination rate was demonstrable The main route of elimination seemed to be active secretion, which was impaired by probenecid. Minor routes of elimination were 3-O-methylation to homovanillic acid and, possibly conjugation.

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Effect of Probenecid on the Levels of Monoamine Metabolites in the Rat Brain

By

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Active transport mechanisms seem to be involved in the removal of organic acids from the cerebrospinal fluid to the blood, as evidenced by perfusion and clearance experiments *in vivo* (PAPPENHEIMER, HEISEY & JORDAN 1961 PROCKOP SCHANKER & BRODIE 1962) The different substances tested, *e g* *p*-aminohippuric acid and diodrast, compete for the same saturable transfer processes, which seem to be similar to those found in the renal tubules.

Probenecid reduces the renal extraction of a variety of organic acids, which includes acid monoamine metabolites such as 5-hydroxyindoleacetic acid (5-HIAA) (DESPOPOULOS & WEISSBACH 1957) and homovanillic acid (HVA) (WERDINIUS 1967 a), derived from the amines 5-hydroxytryptamine (5-HT) and dopamine (DA), respectively These amine metabolites also occur in the brain, with about the same distribution as the corresponding amines (ROOS 1962 ANDÉN ROOS & WERDINIUS 1963 BERNHEIMER 1964) Thus, the greatest amounts of 5-HIAA are found in the brain stem, while HVA occurs predominantly in the corpus striatum.

Noteworthy is the rather low basal level of HVA in the corpus striatum of the rat, about 0.2 µg/g tissue (JUORIO & VOGT 1965 JUORIO SHARMAN & TRAJKOV 1966), as compared to that of DA (3–4 µg/g) In most other species investigated, the normal HVA level is of the order of 2–4 µg/g, *i.e* similar to the DA level. This suggests that active transfer mechanisms might be concerned in the removal of acid monoamine metabolites from the rat brain. It was of interest therefore to investigate whether probenecid, given alone or in combination with reserpine or haloperidol, would interfere with the levels of HVA and 5-HIAA in the rat brain.

) A preliminary report of part of this work has already been published (WERDINIUS 1966).

Materials and Methods

The drugs used were probenecid (dissolved in an equimolar amount of sodium hydroxide, diluted with saline and adjusted to neutrality) reserpine and haloperidol.

Adult hooded rats of either sex weighing 130 to 250 g were used, five animals in each group. In one series of experiments the animals were treated with probenecid (usually 200 mg/kg body wt., i.p.), f llowed after 30 min. by reserpine (10 mg/kg i.p.) or haloperid l (2 mg/kg i p.). Three hours after the last injection the rats were killed and the brains removed. Additional control groups were run with no, or only one, of the drugs for the corresponding time intervals, *i.e.* 3½ hours for probenecid, and 3 hours for reserpine and haloperidol. The animals were kept at an environmental temperature of between 26 and 30° during treatment. The body temperature was checked in some cases no hypothermia occurred.

Brain dissections. The corpora striata (nucleus caudatus and nucleus lentiformis), including some of the underlying orbital cortex, were pooled and analysed for HVA (weight 0.70–1 10 g for five pairs) The remainder of the brains was freed from cortical tissue, and the portions anterior to the caudal colliculi, mainly consisting of the thalamus, hypothalamus and mesencephalon, were pooled (weight 1.20–1 70 g for five such "anterior brai stems") and analysed for 5-HIAA.

Estimation of 5-HIAA and HVA The fluorimetric assays of 5-HIAA and HVA were performed according to Roos (1962) and Andén, Roos & Werdinius (1963), respectively with some modifications. The procedures have recently been described in detail (Werdinius 1967b).

In a few experiments the levels of the corresponding amines were measured, with and without probenecid pretreatment. DA was thus assayed in the corpus striatum by the method of Carlsson & Waldeck (1958) with the modifications described by Carlsson & Lindqvist (1962), and 5-HT by the method of Bertler (1961), modified by Andén & Magnusson (1967). Single rat brains were used for these analyses.

Results

The results are given in table 1 After probenecid pretreatment the level of HVA increased to about three times the low control value of 0.23 µg/g tissue. Roughly the same increase was noted for the group pretreated with reserpine alone (cf Juorio *et al.* 1966) When the two drugs were combined, the HVA level rose nearly tenfold. Similarly haloperidol alone increased the HVA level to more than seven times the control value, and the rise was further augmented by combination with probenecid.

The same general tendency appeared with regard to the 5-HIAA levels, with the exception that haloperidol did not produce a significant increase as compared to the control value. Reserpine and probenecid each increased the level about twofold, and if the drugs were combined, the 5-HIAA increased to about four times the basal level.

The 5-HT level in the brain stem showed no significant change after probenecid pretreatment, nor did the DA level in the corpus striatum.

No significant amounts of HVA or 5-HIAA could be detected in the blood plasma, neither in the treated nor in the control rats.

Table 1

Effect of probenecid (200 mg/kg i.p., 3.5 hours) on the levels of HVA, 5-HIAA, DA and 5-HT in the rat brain, normally or in combination with reserpine (10 mg/kg i.p., 3 hours) or haloperidol (2 mg/kg i.p., 3 hours).

The values are given in μg/g tissue ± s.e.m. Numbers of analyses are given in brackets. Each experiment was made on five pooled brains (for DA and 5-HT one brain).

The probabilities given refer to the effect of probenecid pretreatment in each combination.

	Treatment 30 min. after probenecid		
	No tranquillizer	Reserpine	Haloperidol
HVA (corpus striatum)			
Controls	0.23 ± 0.04 (7)	0.81 ± 0.13 (6)	1.67 ± 0.18 (4)
Probenecid	0.64 ± 0.11 (5)	2.08 ± 0.10 (6)	2.66 ± 0.25 (4)
	$P < 0.005$	$P < 0.001$	$P < 0.02$
5-HIAA (brain stem)			
Controls	0.95 ± 0.03 (5)	2.14 ± 0.33 (4)	1.11 ± 0.13 (4)
Probenecid	1.83 ± 0.27 (4)	3.69 ± 0.50 (4)	1.72 ± 0.09 (5)
	$P < 0.01$	$P < 0.05$	$P < 0.005$
DA (corpus striatum)			
Controls	3.90 ± 0.31 (4)		
Probenecid	3.51 ± 0.53 (4)		
	$P > 0.2$		
5-HT (brain stem)			
Controls	0.55 ± 0.08 (4)		
Probenecid	0.58 ± 0.05 (4)		
	$P > 0.2$		

Discussion

The reserpine-induced increases in HVA and 5-HIAA are probably due, in part, to the release and breakdown of the stored amines (cf. ANDÉN, ROOS & WERDINIUS 1964). DA has been shown to disappear almost completely from the rat brain three hours after administration of reserpine in a dose of 10 mg/kg intraperitoneally (ANDÉN 1967). Similarly, the 5-HT level of the rat brain is markedly reduced after reserpine. The increase in HVA corresponded only to about 15 % of the released DA, indicating either a rapid removal of HVA, or the existence of alternative metabolic pathways for DA. The formation of the alcohols corresponding to the acids should be remembered in this respect: conversion of dopamine to 3,4-dihydroxyphenyl-ethanol has thus been shown to occur in the rat

brain *in vitro* (GOLDSTEIN *et al* 1961) On the other hand, the reserpine-induced increase in 5-HIAA in the brain stem (about 1.2 μg/g or about 6 nM/g) appeared to be in excess of the released 5-HT (at most 0.5–0 6 μg/g or about 3 nM/g). An increased synthesis of 5-HT in addition to release of the stored amine, seems a likely explanation for the increase in 5-HIAA. A third possibility would be an impaired elimination of the metabolite caused by reserpine.

Probenecid caused increases in both HVA and 5-HIAA, which were of the same order of magnitude as those produced by reserpine The amine levels were, however not significantly affected Amine release would thus not explain the increases. An impaired elimination of the metabolites from the brain tissue seems to be probable, even if an increased amine turnover could not be definitely excluded. Combination of probenecid (200 mg/kg) and reserpine caused a considerable further increase in both HVA and 5-HIAA. Smaller doses of probenecid (50 or 100 mg/kg i p) in combination with reserpine caused less marked increases (WERDINIUS 1966) The two drugs thus appear to have different site of action.

Haloperidol belongs functionally but not chemically to the same group of major tranquillizers as the phenothiazines, but has much less peripheral effects. The haloperidol-induced increase in HVA in the brain has been attributed to a reduced elimination of the metabolite or probably more important, to an increased amine turnover secondary to receptor blockage (ANDÉN, ROOS & WERDINIUS 1964) Experiments in rabbits have shown that after pretreatment with the catecholamine synthesis inhibitor H 44/68 (methyl ester of DL-α-methyl-*p*-tyrosine, 200 mg/kg), the haloperidol induced increase in HVA was replaced by a moderate decrease (WERDINIUS, unpublished results)

In contrast to reserpine, haloperidol and the phenothiazines do not seem to change the levels of monoamines in the brain or other tissues The increase in HVA caused by these agents thus does not occur at the expense of stored amines. Higher doses of haloperidol (5 mg/kg i.p) did not produce a further increase in the metabolite concentration. Combination with probenecid, on the contrary nearly doubled the HVA level Hence, the HVA increasing mechanisms are probably not identical for haloperidol and probenecid.

Confirming earlier investigations in the rabbit (ANDÉN, ROOS & WERDINIUS 1964) haloperidol did not increase the concentration of 5-HIAA in the rat brain.

The most probable explanation for the probenecid-induced increases in the brain levels of acid monoamine metabolites seems to be a reduced elimination rate of these substances from the brain tissue. This suggests the existence of an active secretion mechanism which normally removes

the acids from the brain tissue, and which is inhibited by probenecid. Such a transfer mechanism would thus be similar to the well known mechanism in the renal tubules for active secretion of organic acids into the urine.

Whether the elimination from the brain normally proceeds directly into the blood, or partly via the cerebrospinal fluid (CSF), has not been settled In any case, a marked increase in the CSF content of HVA has been demonstrated in the dog after administration of haloperidol as well as of probenecid (ANDERSSON & WERDINIUS, unpublished results)

The proposed mechanism in the rat brain seems to operate at a high speed, as indicated by the very low basal values of HVA. Attempts to reproduce the probenecid effects in the rabbit, with a much higher normal value of HVA in the corpus striatum, were not successful. Perhaps the active transfer mechanism, if present in the rabbit, has such a low capacity that inhibition does not significantly alter the metabolite levels.

Summary

Rats, pretreated with probenecid (200 mg/kg i.p., 3.5 hours previously) showed a two- to threefold increase in the brain levels of the acid monoamine metabolites homovanillic acid and 5-hydroxyindoleacetic acid. Similarly the reserpine or haloperidol-induced increases in the metabolite levels were augmented by pretreatment with probenecid

It is suggested that there is an active secretion mechanism, which removes acid monoamine metabolites and possibly other organic acids from the rat brain, and which is inhibited by probenecid

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Studies on the Metabolism and Elimination of Dopacetamides

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(Received August 10, 1966)

In recent years several substances have been available which block the synthesis of the biogenic monoamines dopamine, noradrenaline, adrenaline and 5-hydroxytryptamine Among these substances are the dihydroxyphenylacetamides (dopacetamides) of which the 3 4-dopacetamides and the 2,3-dopacetamides have attracted the greatest interest.

Of the 3 4-dopacetamides, the α propyl derivative (H 22/54 fig. 1) has been shown to be a highly efficient inhibitor of monoamine biosynthesis (CARLSSON, CORRODI & WALDECK 1963) Several experiments have been performed using this substance, both *in vitro* (NAGATSU LEVITT & UDENFRIEND 1964 BURKARD GEY & PLETSCHER 1964 ROSS & HALJASMAA 1964) and *in vivo* (CARLSSON, CORRODI & WALDECK 1963 CORRODI 1966) The substance was found to inhibit the first steps in the monoamine biosynthesis, *i.e* the aromatic hydroxylations H 22/54 and other 3 4-dopacetamides also block the catecholamine degradating enzyme catechol O-methyltransferase (COMT) (CARLSSON LINDQVIST FILA HROMADKO & CORRODI 1962 CARLSSON CORRODI & WALDECK 1963)

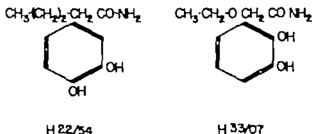


Fig. 1 Chemical structure of H 22/54 and H 33/07

The 2,3-dopacetamides also block the biosynthesis of biogenic amines, but show no inhibition of COMT (CARLSSON & CORRODI 1964). This is of great importance in experimental studies of amine biosynthesis since no side effects (adrenergic hyperactivity) due to inhibited amine catabolism occur. Of these substances, the α-ethoxy derivative (H 33/07, fig. 1) proved to be one of the most active.

It appeared to be of interest to study the metabolism of these compounds, and this paper deals with the rate of elimination of H 22/54 and H 33/07 in different species. In addition, the tissue distribution and metabolism of H 22/54 has been investigated in some detail.

Materials and Methods

Rabbits, rats, cats, dogs and monkeys were used. The monkeys and some of the cats *were anaesthetized with pentobarbitone during the experiment.* The test compound (H 22/54 or H 33/07) was administered in various doses (50–800 mg/kg) by the intravenous (i.v.) or oral (p.o.) route. In some of the rabbit experiments, the animals were pretreated with probenecid, 100 mg/kg i.v., or with the COMT inhibitor H 17/27 (4-tropolone-acetamide), 100 mg/kg i.v. one hour before the H 22/54 injection. All the compounds were dissolved in saline and adjusted to neutrality. Samples of blood (0.5–1 ml) were drawn regularly during the following –4 hours at 15–30 min intervals. The first sample was taken just before the injection, and all blood samples were collected in test tubes containing 0.1–0.2 mg (10–20 IE) dry heparin. The blood samples were stored in the refrigerator and analysed immediately at the end of the sampling period.

In some of the rabbit experiments, the animals were killed at various intervals (0.5 to 3 hours) after the injection of H 22/54 (200 mg/kg i.v.), and the organs (brain, heart, liver, kidney) analysed by means of the paper or column chromatographic techniques described below. In a few additional experiments in rabbits given H 22/54 50 mg/kg the urine was collected and analysed. The animals in these experiments were anaesthetized with urethane (?00 mg/kg i.v.) and catheters were inserted into the ureters for urine sampling.

Assay of H 22/54 and H 33/07 in blood and plasma. The compounds tested (H 22/54 and H 33/07), being catechol derivatives, were assayed fluorimetrically by condensation with ethylene diamine (NATELSON, LUGOVOY & PINCUS 1949, KÄGI, BURGER & GIGER 1957, WEIL-MALHERBE 1959). The details of the analysis were essentially the same as in the procedure for 3,4-dihydroxyphenylacetic acid (DOPAC) described elsewhere (WERDINIUS 1967). The final fluorescence was read in an Aminco-Bowman spectrophotofluorometer against a standard of the test compound. The activating and fluorescent wave-lengths used were 390 and 505 mμ for H 22/54, and 400 and 505 mμ for H 33/07 (uncorrected instrumental values). In each series of blood samples, two or three internal standards were carried through the entire procedure to check the recovery (generally 90–110 %), and the results were corrected accordingly.

Assay of H 22/54 and its metabolites in urine. After dilution 1 : 100 to 1 : 1000 with water 2 ml of the urine were mixed with 0.8 ml of ethylene diamine reagent (ethylene diamine and 4 N ammonium chloride in the proportions 1 : 1.3). The mixture was heated at 63 for 20 min. and then cooled. The fluorescence was read at 390 mμ activating and 505 mμ fluorescent wave-lengths (uncorrected instrumental values), against a standard of H 22/54. Urine which was collected immediately before the H 22/54 administration served as "blank".

For the assay of the total amount of H 22/54 (free and conjugated), the same procedure was used after acid hydrolysis of the urine (3 N HCl, 100 1 hour). Since the amide was converted by the hydrolysis to the corresponding acid, α-propyl-3 4-dihydroxyphenylacetic acid, the final fluorescence was read against a standard f this compound, at the activating and fluorescent wave-lengths 415 and 545 mμ, respectively The recovery of added H 22/54 was 95–100 %.

The 3-O-methylated analogue of H 22/54, α-propyl-3-methoxy-4-hydroxyphenylacetamide, was assayed on urine dilutions by the fluorimetric method of Andén, Roos & Werdinius (1963) which was originally described for homovanillic acid. The fluorescence was read at 315 mμ activating a d 425 mμ fluorescent wave-lengths (uncorrected) against a standard f the methylated amide or for the hydrolysed urine samples, the corresponding acid (α-propyl-homovanillic acid)

Chromatographic demonstration of H 22/54 and its metabolites in tissue The tissue (5 to 10 g) was homogenized in 10 % meta-phosphoric acid (10 ml/g tissue) After centrifugation, filtration and neutralization to pH of about 8, the extract was washed with heptane, saturated with sodium chloride, and then extracted with two 100 ml portions of ether The ether volumes were combined, and the salt-saturated aqueous phase was acidified with 6 N-HCl t a pH of about 1 and re-extracted with ether (2 × 100 ml) for possible acid metabolites. Each of the two combined ether volumes was evaporated at reduced pressure to 50 ml, frozen for one hour in a cold bath (−70°) to remove traces of water, and filtered while still cold. The ether was then evaporated to a small volume and subjected to paper chromatography The solvent used was chloroform (formamide saturated)-glacial acetic acid-*tert*-butanol 25 1 2. The papers (Munktell 8 302) were previously soaked in 20 % (v/v) formamide in acetone, and dried at room temperature. The chromatograms were run for 6 to 8 hours. The reference compounds were H 22/54, its 3-O-methylated analogue, and the corresponding acids formed from these amides by deamidation. After drying at 120 for 5 min. the papers were developed with diazotized *p*-nitroaniline.

Quantitative determination of H 22/54 in tissues In order to separate H 22/54 from interfering tissue material, the amide was adsorbed on to alumina by means of column chromatography The alumina (Aluminium oxide, Merck) was pretreated with acid (2 N-HCl, 100° 30 min.), rinsed several times with water and stored until used. Columns of alumina (35 × 5 mm) wer prepared in glass tubes, and before use they were treated with 20 ml of 0.2 N sodium acetate, followed by 10 ml of water.

The tissue (1 to 4 g) was homogenized in 15 ml 0.4 N perchloric acid containing 1.0 mg of ascorbic acid per ml. After centrifugation and filtration, the extract was cooled to 0° neutralized to about pH 8 with 5 N potassium carbonate, and centrifuged at 0° to remove the potassium perchlorate formed. The clear supernatant was then passed through previously prepared column of alumina. After rinsing with 10 ml distilled water the columns were eluted with 10 ml 0.5 N HCl. The eluate was neutralized to pH 7 2 ml of which were mixed with 0.8 ml ethylene diamine reagent, and the fluorescence developed as described bove for urine. A piece f organ of the same weight, taken from an untreated animal and treated in the same way served as a "blank" Spectra agreed closely with those of a standard solution of H 22/54. The recovery throughout the procedure, as checked by internal standards, ranged between 80–95 % for brain and heart tissue. For liver and kidney some interfering material reduced the recovery considerably and no reliable results were btained.

Results and Discussion

After administration of H 22/54 to the different animal species, there were at first signs of adrenergic hyperactivity such as mydriasis, exophthalmos and increased pulse rate. This hyperactivity is probably due to the COMT inhibiting activity of H 22/54 with subsequent impaired inactivation of the endogenously released adrenergic transmitter noradrenaline. The animals given higher doses of the compound showed signs of reduced sympathetic activity with ptosis and, in the non-anaesthetized animals, sedation within 30 to 60 min. In contrast to this, the animals treated with H 33/07 which is not an inhibitor of COMT did not exhibit the initial phase of hyperadrenergic activity: they were immediately sedated. Dogs and rats exhibited only slight and transcient signs, while the signs were much more prolonged in cats.

Considerable amounts of H 22/54 and H 33/07 could generally be demonstrated in the blood during the hours following their intravenous administration. The concentration of the substances was almost the same in whole blood and in plasma. A fraction of the amides thus seemed to penetrate into the erythrocytes. Apparently the amide content of the erythrocytes rapidly re-equilibrated with the saline, with which the blood was mixed in the first step of the analysis. Essentially the same results were obtained if the erythrocytes were hemolyzed (saline exchanged for water in the first step), and the levels obtained corrected for the considerably lower recovery. For technical reasons, most of the analyses were performed on whole blood.

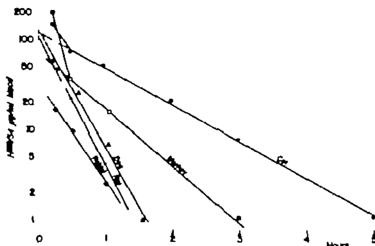


Fig. 2. Blood levels of H 22/54 in different species after the intravenous injection of H 22/54 100 mg/kg (rabbit 50 mg/kg).

Table 1

Apparent half-lives of H 22/54 in the blood of different species.

Species	Dose mg/kg i.v	Half-life min.
Rabbit	50	1[illegible], 17 14 11 11 16, 15
	200	28
	300	32, 19
Rat	50	9
	100	9 12
	200	28
Dog	50	7
	100	12
Monkey	100	37 29
Cat	100	135 47 34 45 48

H 22/54 and H 33/07 were eliminated from the blood stream more or less rapidly and when the concentrations were plotted in a semilogarithmic diagram (log concentration against time) a linear relationship became apparent, particularly at doses of 50 and 100 mg/kg intravenously (fig. 2) The primary elimination routes thus seemed to be of the first order type at these lower doses. The apparent half life of the drug was evaluated from the linear part of the elimination curve, following an initial phase of equilibration between the plasma and the tissues For reasons which are as yet obscure, the apparent half-life was longer at higher doses, and the linearity less well-defined, particularly for H 33/07

The disappearance rate of H 22/54 varied markedly among the species tested and sometimes also within one species. Apparent half lives, as plotted from the linear part of the blood decay curve, are given in table 1 The elimination rate was evidently slowest in the cat. Dogs and rats, on the other hand seemed to be rapid H 22/54 eliminators, whereas monkeys and rabbits showed rates between these extremes. The same general tendency was true for H 33/07 (table 2).

Species differences, as well as individual variations in drug metabolism seem to be common (for review see BURNS 1962) Remarkably one of the cats showed a half life for H 22/54 which was almost three times longer

Table 2

Apparent half-lives of H 33/07 in the blood of different species.

Species	Dose mg/kg i.v.	Half-life min
Rabbit	50	24 16, 22, 24
	300	70, 20, 37
Rat	50	12
	200	15, 14
Cat	300	110

than that seen in other cats. The explanation for this is not clear Strain differences, with regard to elimination mechanisms, might be involved. The possible effect of the anaesthetic (pentobarbitone) should also be considered, since barbiturates are known to activate the microsomal metabolism of some drugs (REMMER 1962)

The compound SKF 525A, which is known to inhibit several types of drug transforming enzymatic reactions in the microsomes (see NETTER 1962), did not significantly affect the half life of H 22/54 in rabbits. Similarly pretreatment with probenecid or with the COMT inhibitor H 17/27 did not affect the half life of H 22/54 or H 33/07

After oral administration of H 22/54 (200 mg/kg) to a dog, no measurable blood levels were detected. However a cat which received H 22/54 100 mg/kg p o showed blood levels of 10 to 20 µg/ml during the first three hours. In view of the widely different half lives in the two species, these findings probably indicate a rapid elimination rather than a poor intestinal absorption in the dog. Similarly even after as high a dose as 800 mg/kg p o to a rat, the blood concentration was at most 10 µg/ml during the first hour and then rapidly declined.

From the quantitative assays (see table 3) and the paper chromatographic experiments, it is evident that H 22/54 penetrates into various organs, such as the brain, heart, liver and kidney In addition to H 22/54 the 3-O-methylated metabolite was demonstrable, by means of paper chromatography in blood plasma and all the organs tested, except the liver No acid metabolites, which could possibly have been generated by deamidation, were detected.

Six hours after administration of H 22/54 (50 mg/kg i v) to rabbits, less than 1 % had been excreted in the free form. However 73 % was recovered

Table 3

H 22/54 (µg/g) in plasma, brain and heart of rabbits at various intervals after the 1.v administration of the drug (200 mg/kg).

T me hours	Plasma	Brain	Heart
0.5	–	12.6	41.3
1	–	7.5	16.6
1	28.5	8.4	8.4
1	–	8.6	25.1
3	–	0.2	5 4
1	25.3	–	–
2	7 4	–	–
3	1.0	0 3	0.9

after acid hydrolysis of the urine More than half of the amount excreted was already present after one hour The 3-O-methylated analogue of H 22/54 appeared in the urine gradually and at six hours represented 22 / of the original amount of H 22/54 administered Most of the metabolite occurred as a conjugate.

From the relative amounts of H 22/54 metabolites in the urine, it appeared that the most important metabolic pathway for H 22/54 is conjugation, at least in the rabbit The nature of the conjugate was not investigated. Glucuronide formation is, however probable. Cats are known to lack the ability to form glucuronides (DUTTON & GREIG 1957 ROBINSON & WILLIAMS 1958) and this deficiency could quite well explain the slow elimination rate of H 22/54 in the cat.

Another pathway of inactivation of H 22/54 seems to be 3-O-methylation. That the liver lacked the 3-O-methylated metabolite, in spite of the high concentration of COMT in this organ, might possibly be due to further conversion of the substance, *e.g* conjugation. However since inhibition of COMT did not slow the elimination rate of H 22/54 in the rabbit, 3-O-methylation seems to be only an alternative metabolic route, and is not rate-limiting at lower doses.

The inhibition of the catecholamine degradating enzyme COMT which is a conspicuous feature of the 3 4-dopacetamides, is probably due to the fact that H 22/54 itself forms a substrate for the enzyme. From *in vitro* experiments it would appear that 2,3-dihydroxyphenolic compounds are poor substrates for COMT (MASRI BOOTH & DE EDS 1962), and H 33/07 is probably metabolized in ways other than by methylation, *e.g* by conjugation. The metabolism of H 33/07 has not as yet been investigated in detail.

When different catechol compounds in the blood are considered from a metabolic point of view certain differences appear The circulating catecholamines are rapidly inactivated, partly by an active uptake into the peripheral adrenergic neurons, partly by 3-O-methylation after the action of COMT (AXELROD 1960) The acid catecholamine metabolites (such as 3 4-dihydroxyphenylacetic acid and homovanillic acid) are principally eliminated by means of active renal secretion and, at least in the rabbit, mainly in the free form (WERDINIUS 1967) The amides (such as H 22/54) which are closely related to these acids, seem to be principally conjugated, a process which results in more polar and more easily excreted compounds.

Summary

The metabolism and elimination of two compounds interfering with the biosynthesis and metabolism of catecholamines were studied. Both compounds belonged to the dopacetamide series α-propyl 3 4-dihydroxy phenylacetamide (H 22/54), and α-ethoxy-2,3-dihydroxyphenylacetamide (H 33/07).

After intravenous administration (50 to 100 mg/kg) the drugs were eliminated from the blood apparently by first order kinetics.

Considerable species differences were noted in the elimination rate of the amides. Rats and dogs were the most rapid eliminators, cats the slowest, whereas rabbits and monkeys were between these extremes. The two substances were similar in this respect.

The most important pathway of elimination for H 22/54 was conjugation, though 3-O-methylation was also found. The elimination rate was not affected by substances such as probenecid, the microsomal inhibitor SKF 525A, or the catechol-O-methyltransferase inhibitor 4-tropolone-acetamide.

Physico-chemical properties seem to be of great importance for the metabolic conversion of different catechol compounds. The structure of the side chain apparently determines the metabolic fate of the compound.

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The Oral Toxicity in Mice and the Uptake by Diphyllobothrium Latum and the Host Gut of some Anthelmintics in Vitro

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In spite of the high toxicity of many fern extracts they are still in general use as anthelmintic in Finland, particularly against *Diphyllobothrium latum*. Several pure substances with anthelmintic activity have been isolated from the crude extract and some substances have been synthesized. There is no reliable standardization method for the fern extracts. HUHTALA (1949) found considerable differences in the crude filicin content of the fern extracts of different origins. He found that the crude filicin content of the extracts showed no correlation to their toxicity in earthworms and only a limited correlation to their subcutaneous toxicity in mice. Comparative studies on the mammalian oral toxicity of anthelmintic drugs have not been reported.

Very little is known about the absorption of anthelmintics by *Diphyllobothrium latum* and nothing about the factors which affect it. The uptake of niclosamide by the rat tapeworm *Hymenolepis diminuta* has previously been studied by STRUFE & GÖNNERT (1960)

In a previous study we compared the effect of different anthelmintics on some metabolic functions of the cat s tapeworm *Taenia taeniaformis in vitro* (MATTILA & TAKKI 1966) The purpose of the present investigation was to compare the uptake of niclosamide and desaspidin by *Diphyllobothrium latum* and by the intestinal tissue of the host *in vitro*, as well as to determine the oral toxicity in mice of the same drugs and of different fern extracts.

Methods

In vitro experiments *Diphyllobothrium latum* was grown in dogs by infecting them with infected lake fish of the tapeworm area of Middle Finland. Dogs under pentobarbital anesthesia, were allowed to bleed to

death. The jejunal and ileal gut was opened and tapeworms were re moved. Pieces of 300–700 mg of tapeworm were incubated by shaking in 10 ml volume of Krebs-Ringer phosphate buffer solution, pH 8 0–8.1 containing the drugs, for 30 min. at 37 Pieces of 100–300 mg the ileum of host dogs were similarly incubated in Krebs Ringer phosphate buffer In other experiments, pieces of 5 cm of the ileum were closed at both ends and the drugs, dissolved in 3 ml of the same buffer solution, were injected into the lumen these pieces were then incubated in a 20 ml volume of another Krebs-Ringer phosphate buffer pH 7 4–7.5 After the incubation the pieces were washed with the buffer solution and homogenized. The closed blocks of ileum were opened and washed, the mucosal and muscular layers separated and cut into pieces of 300–500 mg, which were then homogenized. Nine volumes of acetone were added and the samples centrifuged The supernatant was drawn off and evaporated *in vacuo* The dried residue was dissolved in 1 ml of ether-alcohol-water (1 1 1) solution and the niclosamide concentration determined after ad dition of ethanolamideacetone at 395 nm according to STUFE & GÖNNERT (1963) For the determination of desaspidin, the same volume of 0 1% aqueous "Echtblausalz B solution was added to the ether-alcohol-water extracts. The absorption was measured at 480 nm, which is the maximum point of the color spectrum of samples and pure desaspidin. At other wavelengths the spectra run slightly differently All readings were made against a tissue blank and a reagent blank. Thin layer chromatography (BLAKEMORE *et al* 1964) and paper chromatography (Penttilä & SUNDMAN 1961) were used for separation of the drugs and their metabolites.

White male mice weighing 20–22 g were used for the toxicity studies. The mice were fasted for 10 hours before the experiments but received water *ad libitum* Niclosamide (yomesan ®, Bayer AG Leverkusen) desaspidin and flavaspidic acid (OY Medica AB., Helsinki) and two kinds of fern extracts (Alb Koponen, Nurmijärvi) were used both these extracts are mentioned in The Finnish Pharmacopoeia (Ph. F VII) Ex tractum filicis (from *Dryopteris filix-mas)* and Extractum dryopteridis (from *Dr austriaca* or *Dr euspinulosa)* In addition one Danish fili extract (Pharmacopoeia Danica 1948) and one extract mixture from Russian or/and Balkanian ferns (Extr filicis maris aether Blembel Ge brüder Hamburg) were used. All the drugs were dissolved in 1 % sodium cholate solution. The pH of the solution was adjusted to between 7.5 and 7 9 with sodium hydroxide. The drugs were administered into the stomach of the mice by means of a thin polyethylene cannula, in a volume of less than 0 5 ml The mice were placed in plastic cages, one in each cage, and results observed after 24 hours. After preliminary tests, 3 or 4 groups of 9 to 10 mice were used for each drug, and control tests with

Table 1

Desaspidin content in pieces of Diphyllobothrium latum and the ileum of its host dog, after 30 min. incubation in Krebs-Ringer phosphate buffer solution containing 0.[illegible] mg/ml of desaspidin. Other substances were added to this sol tion.

Tissue	Substances added into the phosphate buffer in per cent f the total volume	Desaspidin content µg/g of wet tissue. Mean ± s.e.m. Number of estimation in brackets	
Tapeworm	Control (desaspidin in buffer)	(5)	36.74 ± 6.04
	Alcohol 1 %	(5)	30.32 ± 13.21
	Tween 80 5 %	(4)	5.01 ± 1.34
	Castor oil 5 %	(4)	23.72 ± 9.21
Dog ileum	Control (desaspidin in buffer)	(4)	42.93 ± 5.81
	Alcohol 1 %	(4)	59.58 ± 24.67
	Tween 80 5 %	(3)	6.53 ± 2.29
	Castor oil 5 %	(3)	30.56 ± 9.76

Table 2

Niclosamide content in pieces of Diphyllobothrium latum and the ileum its host dog fter 30 min. incubation in Krebs-Ringer phosphate buffer solution containing 2 mg/ml of niclosamide. Other substances were dded t this solution.

Tissue	Substances added into th phosphate buffer in per cent of the total volume	Niclosamide content µg/g of wet tissue. Mean ± s.e.m. Number of experiments in brackets	
Tapeworm	Control (niclosamide in buffer)	(3)	10.16 ± 4.30
	Alcohol 1 %	(3)	11.92 ± 6.67
	Tween 80 5 %	(3)	10.95 ± 4.20
	Liquid paraffin 5 %	(2)	5.04 ± 0.92
Dog ileum	Control (niclosamide in buffer)	(5)	6.60 ± 2.61
	Alcohol 1	(6)	7.46 ± 2.01
	Tween 80 5 %	(6)	5.39 ± 0.87
	Liquid paraffin 5 %	(6)	8.38 ± 3.23

desaspidin were repeated if necessary to rule out variations in groups of mice. The LD50 was calculated using the graphical method of Miller and Tainter (1944)

Results

A. *In vitro experiments*

Tables 1 and 2 show that the dog's intestinal wall and the tapeworm tissue showed about an equal uptake of anthelmintic drugs from the

Table 3

The uptake of niclosamide and desaspidin by the ileal tissues of dogs as modified by ethyl alcohol. The pieces of ileum were closed at the both ends, drugs were injected into the lumen and the gut pieces were incubated for 30 min. in Krebs-Ringer phosphate buffer solution. The number of estimations in brackets.

Buffer in gut lumen	Tissue	Desaspidin content µg/g of wet tissue Mean ± s.e.m.		Niclosamide content µg/g of wet tissue Mean ± s.e.m.	
Control	Mucous layer	(6)	15.25 ± 4 13	(5)	3 16 ± 0.41
Alcohol 5 %		(6)	5 04 ± 0.60	(6)	3 19 ± 0.31
Control	Muscular layer	(6)	2.91 ± 0 71	(6)	2.46 ± 1 10
Alcoh l 5 %		(5)	1 58 ± 0.09	(6)	2.26 ± 0.21

incubation medium. The uptake of desaspidin by the host gut and the tapeworm tissue was three times higher than that of niclosamide, even though the concentration of desaspidin in the incubation medium was only one tenth that of niclosamide.

In all experiments except one, ethyl alcohol tended to increase the uptake of the anthelmintic drugs by the host tissue, but because of the high standard deviation this tendency was not statistically significant. In the experiments in which alcohol and the anthelmintics were put into the gut lumen, alcohol clearly decreased the uptake of desaspidin but not that of niclosamide (Table 3) Tween 80 markedly decreased the uptake of desaspidin by the dog s intestine and the parasite tissue, but it did not modify the uptake of niclosamide. Castor oil did not significantly inhibit the uptake of desaspidin both by the host and parasite tissue. Liquid paraffin seemed to decrease the uptake of niclosamide by the tapeworm but not by the intestine of the host.

When different concentrations of anthelmintic drugs were used in the incubation fluid, the recovery of the drugs from the tapeworm was always linear From the dog s intestine, however improved recoveries of the drugs were obtained using higher drug concentrations. The rapid biotransformation of desaspidin by the dog s intestine was demonstrated by using the paperchromatographic method of PENTTILÄ & SUNDMAN (1961) and thin layer chromatography according to BLAKEMORE *et al.* (1964) Both methods revealed a formation of several other phlorobuty rophenone derivatives during incubation of the dog s intestine, but much less during incubation of the tapeworm.

B *Toxicity studies*

Table 4 shows the acute oral toxicity of fern extracts and two pure fern substances. Desaspidin was twice as toxic as flavaspidic acid The

Table 4

The acute oral toxicity in white mice of desaspidin, flavaspidic acid and different samples of fern extracts. The LD50 of fern extracts is calculated for their crude filicin.

Drug	LD50 ± s.e.m. mg/kg
Desaspidin	340 ± 48 mg/kg
Flavaspidic acid	690 ± 158 –
Desaspidin + flavaspidic acid (1 1)	*520 ± 46* –
Filix extracts (PhF VII)	
1956	580 ± 61 –
1958	605 ± 72 –
1960	550 ± 67 –
1961	580 ± 51 –
Dryopteris austriaca extracts (PhF VII)	
1954	460 ± 47 –
1955	305 ± 38 –
1959	320 ± 34 –
1962	260 ± 39 –
Danish filix extract (PhD 48)	350 ± 43 –
Unknown fern extract from Russia and/or Balkans (content of crude filicin 22 per cent)	380 ± 24 –

LD50 of a mixture (1 1) was similar to that of filix extracts and thus the mixture can be used as a standard for the toxicity studies of filix extracts. The Danish filix extract was almost as toxic as the Finnish *Dryopteris austriaca* extracts. The unknown fern extract mixture originated from Russia and/or the Balkans was of about the same toxicity corresponding to the Finnish *Dryopteris austriaca* extracts. These more toxic extracts in thin layer chromatography showed a red spot (Rf 0 14), running at the same speed as butyrofilicinic acid. This spot was not detected in chromatograms prepared from the Finnish filix extracts. No clear difference was found in the paper chromatograms prepared from the least toxic and the most toxic sample of *Dryopteris austriaca* extracts.

Castor oil, ethyl alcohol and especially Tween 80 increased the toxicity of desaspidin (Table 5) Alcohol, however did not increase the content of desaspidin or its metabolites in the whole mouse when two desaspidin-treated and two alcohol plus desaspidin treated mice were homogenized after gastroenterectomy and desaspidin and its metabolites were analysed by thin-layer chromatography Because of the low acute toxicity and the poor solubility of niclosamide it was not possible to determine its oral LD50 in mice, with or without tween 80, castor oil or alcohol

Table 5

The acute oral toxicity in mice of desaspidin and niclosamide given as a cholate suspension, and the effect of added castor oil, alcohol and Tween 80.

Drug	LD50 ± s.e.m. mg/kg
Desaspidin	340 ± 48 mg/kg
– + 10 alcohol	155 ± 29 –
– + 10% castor oil	180 ± 29 –
– + 10% Tween 80	49 ± 45 –
Niclosamide	>2000 –
– + 10% alcohol	>2000 –
– + 10% castor oil	>2000 –
+ 10% Tween 80	>2000 –

Discussion

The toxicity of the Danish filix extract differed from that of Finnish filix extracts. Thus the common maximal dose for Finnish and Danish filix extracts in the Nordic Pharmacopoeia does not seem to be reasonable, since no standard requirements are stated in the Nordic Pharmacopoeia. The common therapeutic dose for the extracts prepared from *Dryopteris filix mas* or *Dryopteris austriaca* is not justified although it has been used As is obvious from the toxicities of fern extracts of different origin, toxicity studies are necessary to prevent the use of highly toxic extracts with a relatively low content of crude filicin. Although phloroglucinol derivatives are by far the most effective anthelmintic components of fern extracts (Blakemore *et al* 1964), the precise role of the different components is not known.

Dryopteris austriaca extract contains polycyclic phloroglucinol derivatives (Penttilä & Sundman 1963a & b). Considerable differences in the toxicity of various *Dryopteris austriaca* extracts were found and they also differed in their effect on respiration and anaerobic glycolysis of *Taenia taeniaformis* tissue *in vitro* (Mattila & Takki 1966)

The *in vitro* experiments clearly showed that there was a lower absorption of niclosamide than of desaspidin both in host ileum and parasite. This was probably due to the very poor solubility of niclosamide, in agreement with its lower oral toxicity as well as with the need for a ten times higher anthelmic dose On the other hand, the toxicity of niclosamide after intravenous administration is similar to that of desaspidin (Östling 1962)

The 30 min. values of the *in vitro* experiments may reflect mainly the

binding capacity of the host intestine as well as of parasite tissue and not the rate of absorption. This would explain the odd effects of tween 80 and alcohol on the absorption of desaspidin Both these substances increased the oral toxicity of desaspidin in mice but decreased its content in dog's ileum *in vitro* We found no evidence that alcohol improved absorption of desaspidin.

Desaspidin and niclosamide inhibit oxydative phosphorylation in the tapeworm and host liver mitocondria and this may well be responsible for the anthelmintic effect (GÖNNERT & SCHRAUFSTÄTTER 1960 RUNEBERG 1963 MATTILA & TAKKI 1966) The present results indicate no great differences in the absorption of the anthelmintics by the parasite and host intestine and their selective anthelmintic effect may to a considerable degree depend on the lower metabolism of drugs in the tapeworm than in the host. An additional factor of cause, is the dilution in the larger tissue volume in the host. The urinary excretion of these poorly soluble drugs as such is probably very slight.

Little is known about the metabolites of the anthelmintic drugs. The nitrogroup of niclosamide is reduced to amino-group (STRUFE 1964) There is no evidence whether the corresponding enzyme is present in *Diphyllobothrium latum* Theoretically both niclosamide and desaspidin as well as other phlorobutyrophenones can form glucosiduronic acids in mammalian tissues, since most phenolic compounds are good substrates for glucuronyl transferase There is considerable glucuronyltransferase activity in the intestinal mucosa of most mammalian species, including man (HARTIALA 1961) *Diphyllobothrium latum* homogenate, on the contrary lacks this glucuronyltransferase activity (HUTTUNEN AIRAKSINEN & TAKKI 1965, unpublished).

Summary

Oral toxicity of three anthelmintic drugs, desaspidin, flavaspidic acid and niclosamide as well as fern extracts of different origin were studied in mice. The uptake of desaspidin and niclosamide by *Diphyllobothrium latum* and the intestine of its host (dog) were compared *in vitro*

Desaspidin was twice as toxic as flavaspidic acid. Castor oil, alcohol and, to an even greater extent, tween 80 increased the toxicity of desaspidin. The oral toxicity of niclosamide was so low that the LD50 could not be determined with or without alcohol or tween 80 There were considerable differences in the toxicity of different fern extracts. Extractum dryopteridis austriacae was nearly twice as toxic as Extractum filicis and a Danish filix extract was more toxic than Finnish extracts.

Uptake of the anthelmintics by the parasite and by the host intestine

was about the same. The selective anthelmintic toxicity seems to depend on the lower metabolism of drugs in the tapeworm than in the host, a fact which has been confirmed chromatographically for desaspidin.

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Non-Specific Reaction of Current Glucose Oxidase Preparations with Glycogen and Its Application for Glycogen Determinations in Tissue

By

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(Received June 13 1966)

During a study on the initial hyperglycaemic phase following alloxan poisoning in mice (RERUP & LUNDQUIST 1967) the question arose concerning rapid changes in liver glycogen. Since blood glucose levels at this laboratory are routinely determined using the coupled enzyme system consisting of glucose oxidase and peroxidase together with a chromogenic oxygen acceptor (MIDDLETON & GRIFFITHS 1957 MARKS 1959) it was decided to try to determine the liver glycogen using the enzymatic reaction instead of the copper reduction after acid hydrolysis of glycogen (GOOD *et al.* 1933) During this investigation it became evident that glycogen reacts quantitatively with the coupled enzyme system of the present degree of purity but that the activity measured decreased rapidly during the early phase of acid hydrolysis. A valid and reproducible procedure for glycogen determinations using the enzyme reaction has been established. This method together with a comparison with other methods, copper reduction after acid hydrolysis and anthrone reaction, is reported.

Material and Method

Liver glycogen was determined in female non-fasting mice of the NMRI strain kept on standard pellet diet and tap water ad libitum.

Purified glycogen was obtained from 1) Merck A.G Darmstadt, Germany and 2) Nutritional Biochemical Corporation Ltd., Cleveland, Ohio, U.S.A. These preparations will be referred to below as standard glycogen.

Standard glycogen was determined 1) by treatment with the anthrone reagent (CARROLL *et al.* 1956) 2) by copper reduction following hydrolysis in 0.6 N HCl at 95° (SHAFFER &

obtained from Laboratory Animal Breeding, Laven, Denmark.

Somogyi 1933) or 3) by direct treatment with glucose oxidase + peroxidase together with o-tolidin (Marks 1959) or in some experiments, with 0-dianisidin (Huggett & Nixon 1957).

Liver glycogen was extracted 1) according to Good *et al* (1933) by boiling the tissue in 30 % KOH followed by precipitation with 1.5 volumes of ethanol the precipitate was dissolved at an acid, neutral, or alkaline pH (about 0.8 7.0 and 12.5), incubated at 80° or 95° and samples taken at different intervals for analysis of a) copper reducing substances or b) in the coupled enzyme system 2) according to Carroll *et al.* (1956) by homogenization of the tissue in 5 % trichloroacetic acid followed by precipitation with 4 or 1.5 volumes of ethanol the precipitate was dissolved in re-distilled water and analyzed a) using the anthrone reaction or b) in the coupled enzyme system.

Glucose was determined by copper-iodometric titration (Shaffer & Somogyi 1933) or in the coupled enzyme system. Glucose oxidase was obtained from 1) Hughes & Hughes, London, as "Fermcozyme" a stable liquid preparation containing 750 units per ml 2) Kabi, Stockholm, as powder 3) Sigma, St. Louis, as a powder Peroxidase was obtained from Boehringer & Soehne, Mannheim. Extinction was measured in Beckman model B spectrophotometer at 420 mμ (o-dianisidin reaction) or 625 mμ (o-tolidin reaction) for routine analysis an EEL colorimeter using filter OB10 and special filter 607 respectively was used *The method adopted for glycogen determination in tissue (mouse liver) is as follows*

Weigh glass stoppered centrifuge tubes containing 2 ml of 30 % KOH Immediately after killing the mouse, transfer liver specimen (20–100 mg about) into the tube. Stopper and weigh. Incubate the tube in a boiling water bath until the tissue has completely disintegrated (3–6 minutes). Remove from water bath, add 3 ml of pure ethanol, incubate until the mixture boils and cool at room temperature. After cooling, centrifuge at about 1000 g for 5 minutes, decant the supernatant and allow to drain. Incubate the precipitate for one minute in the boiling water bath, in order to expel any remnants of ethanol. Dissolve the precipitate in re-distilled water which usually yields a pH of about 12.5. The volume of water added depends on the expected liver glycogen level, i.e. in normal non-fasting mice 1 ml/10 mg of wet liver yielded glycogen concentrations suitable for colorimetry if 25 or 50 μl was added to 3 ml of the reagent containing glucose oxidase, peroxidase, and o-tolidin in an acetate buffer at pH 5 as described by Marks (1959). Standard glycogen is used as reference and the amount of glycogen in the sample calculated. The result is expressed as per cent glycogen in terms of wet liver

Results

In order to determine the time necessary to obtain hydrolysis, standard glycogen was incubated in 0.6 N HCl (Good *et al* 1933) Since in the original paper the temperature was not specified, we tried to hydrolyze at 80° and analyzed samples at different time intervals for activity in the coupled enzyme system (fig. 1).

The result (fig. 1) shows that measurements of material expected to be glucose did not increase with time – as first assumed – but decreased to about half the value measured at zero time. It was thus demonstrated that non-hydrolyzed glycogen yields a positive reaction in the coupled enzyme system. This finding was confirmed with mouse liver extracted glycogen precipitate Standard glycogen was then dissolved in re-distilled

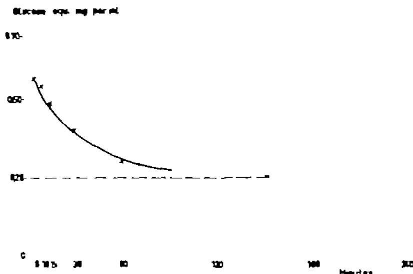


Fig. 1 Glycogen (Merck), 0.5 mg/ml in 0.6 N HCl incubated at 80° Analysis of 25 µl of incubate at different times in the coupled enzyme system. Comparbo with glucose (0.5 mg/ml). Abscissa Time in minutes Ordinate Glucose equivalents, mg/ml.

water or 0.01 N NaOH (0.5 mg/ml) and analyzed immediately In each case glycogen yielded a reaction which was equivalent to about 0.55 mg/ml of glucose in mutarotation equilibrium. The same was true when standard glycogen from a different source (see method) was used. Both purified glycogen preparations showed the same potency The figure of equivalence of 0.5 mg/ml of glycogen and 0.55 mg/ml of glucose was obtained with the particular enzyme preparation (Fermcozyme) used in

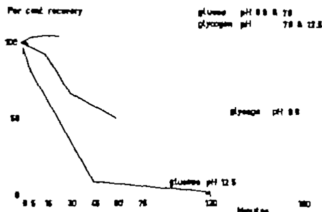


Fig. 2. Effect of pH on stability of glucose and glycogen t 80° Abscissa Time in minutes Ordinate: Activity in the coupled enzyme system, expressed as percentage of the initial.

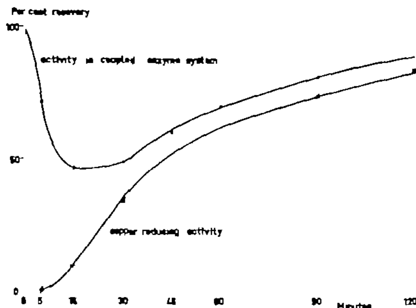


Fig. 3 Hydrolysis of glycogen in 0.6 N-HCl at 95°. Abscissa: Time in minutes. Ordinate: Percentage recovery, calculated from amount of glycogen incubated at zero time for copper reducing activity, and expressed in terms of initial reading for activity in the coupled enzyme system.

these experiments and varied with different glucose oxidase preparations. An interpretation of this is given in the discussion.

Stability of glucose at low pH and of glycogen at high pH was checked by incubating standard glycogen and glucose at 80° in 0.6 N HCl, 0.33 M phosphate buffer and 0.01 N NaOH (pH values: about 0.8, 7.0 and 12.5) respectively.

As shown in fig. 2 glucose was rapidly destroyed in alkaline medium, whereas glycogen was unstable at low pH; under the remaining conditions both substances were stable during the experiment. However, while glucose was broken down entirely at pH 12.5, the measured activity of glycogen at low pH approached asymptotically about half the value at zero time. This was further investigated in an experiment in which the temperature was raised to 95° and both copper reducing activity (Shaffer & Somogyi 1933) and activity in the coupled enzyme system were measured.

It can be seen from fig. 3 that copper reducing activity increased with time as expected from acid hydrolysis of glycogen. The measured activity in the glucose oxidase-peroxidase system, however, was again highest at zero time, fell (more rapidly than at 80°, see fig. 1) to about half the initial value during 15 minutes and began to rise slowly after 30 minutes,

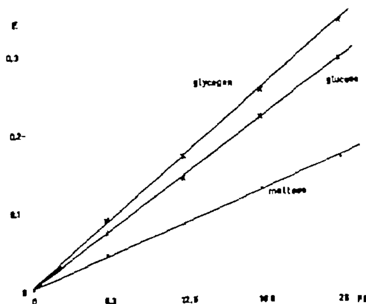


Fig. 4a. Agreement with Beer's law of glycogen and maltose as well as of glucose in the coupled enzyme reaction. Abscissa: Added amount of active compound in µg. Ordinate: Measured extinction.

roughly in parallel with the activity measured by copper reduction. Several repetitions of this experiment fully confirmed these results and showed that even under prolonged hydrolysis, the final measurements in the coupled enzyme system never reached the values recorded at zero time.

Of other polysaccharides tested only soluble starch, but not gum arabic, hyaluronic acid, or dextran, gave a positive response, whereas of the disaccharides tested, viz. saccharose, lactose, trehalose, and maltose, only maltose yielded a clear reaction (about half the extinction measured in terms of glucose). Maximal extinction after addition of maltose to the enzyme system was, however, observed much later than after 10 minutes from mixing (peak readings at about 15–16 minutes instead of the usual 10 minutes), which precluded any valid quantitative comparisons.

Agreement with Beer's law of the reaction in the coupled enzyme system with glycogen and maltose is shown in fig. 4a; the pattern of colour development is given in fig. 4b.

From the above observations it appeared likely that during acid hydrolysis, glycogen leads to a positive reaction in the coupled enzyme system in three phases: 1) Initially as non-hydrolyzed glycogen. 2) Mainly as maltose in the early phase of hydrolysis. 3) As glucose in the late phase of hydrolysis.

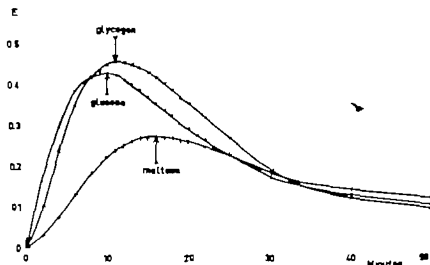


Fig. 4b. Colour development pattern of glucose, glycogen, and maltose in the coupled enzyme system using o-tolidin as chromogenic oxygen acceptor Abscissa Time in minutes Ordinate Measured extinction Arrows indicate time of maximal colour intensity

In order to obtain further evidence in support of this idea, glycogen and maltose were incubated in 0 6 N HCl at 95 and the activity in the coupled enzyme system was followed for 7 hours. Moreover the colour development pattern of a glycogen hydrolysate (15 minutes at 95° in

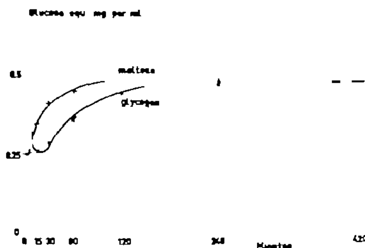


Fig. 5a. Acid hydrolysis of glycogen and maltose at 95 in 0.6 N HCl. Abscissa Time in minutes Ordinate Measured activity in the coupled enzyme system, expressed as glucose equivalents (mg/ml)

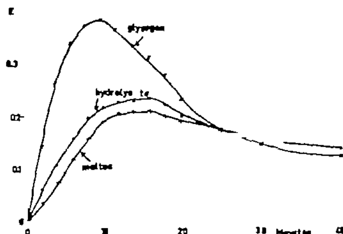


Fig. 5b. Comparison of colour development pattern of glycogen, glycogen hydrolysate (15 minutes exposure to 95° in 0.6 N HCl), nd maltose. Abscissa Time in minutes Ordinate Measured extinction.

0.6 N HCl, see fig. 3) was compared with non-hydrolyzed glycogen and maltose The results appear in fig. 5a and 5b.

As shown in fig. 5b the colour development of partly hydrolyzed glycogen was almost identical with that of maltose. Fig. 5a shows that the increase of active material in the coupled enzyme reaction during hydrolysis proceeded with almost the same velocity either following incubation of maltose or during the second phase of glycogen hydrolysis. The findings also indicate that hydrolysis converts glycogen into maltose at a higher rate than maltose into glucose, and that a higher temperature is necessary to achieve the latter conversion, under the condition stated.

The above findings show the possibility of using the coupled enzyme system for the quantitative determination of glycogen. For this reason a comparison was made between known methods of liver glycogen determination using a) KOH extraction and determination of copper reducing activity after acid hydrolysis of the ethanol precipitate (GOOD *et al* 1933) or b) trichloroacetic acid extraction and determination of the ethanol precipitate by means of the anthrone reaction (CARROLL *et al* 1956) or either of the above extraction procedures followed immediately by analysis in the coupled enzyme system of the ethanol precipitate after dissolving at alkaline pH (about 12.5) The results are given in table 1 and table 2.

Comparison of the methods showed satisfactory agreement between the two methods confirming the results of CARROLL *et al* (1956) Determinations of glycogen by means of the coupled enzyme system, however

Table 1

Comparison of mouse liver glycogen determinations using a) the glucose oxidase-peroxidase system without acid hydrolysis and b) copper reduction (SHAFFER & SOMOGYI 1933) following acid hydrolysis. Glycogen was extracted in 30 % KOH and precipitated with 1.5 volumes of ethanol (GOOD *et al.* 1933)

Mouse	Glycogen, per cent of wet liver determined		a/b per cent
	a) by enzymes	b) by copper reduction after hydrolysis	
1	3.01	2.89	104
	1.84	1.22	151
3	2.41	2.07	116
4	2.11	1.70	124
5	1.54	1.22	124
6	5.44	4.35	125
7	4.36	3.16	138
8	3.67	3.58	103
9	3.99	2.71	147
10	1.93	1.80	107
11	2.31	1.77	131
12	2.23	1.99	112
13	1.46	1.40	104
Mean	2.79	2.30	122

Analysis of variance	Mean square	F	P
Mice	2.34205	24.98	<0.001
Methods	1.58031	16.85	<0.005
Error	0.09376		

Correlation = 0.948 s_r = 0.09593 t = 9.88 P = <0.001

yielded slightly but significantly higher values (about 20 %) than either of the known methods

Experiments with different glucose oxidase preparations

In order to determine whether contamination of glucose oxidase might be responsible for the observed positive reaction of glycogen and maltose in the coupled enzyme system, two different glucose oxidase preparations were used instead of the usual one (Fermcozyme) The colour development pattern of equal weight/volume concentrations of glucose, maltose,

Table 2.

Comparison of mouse liver glycogen determinations using a) the glucose oxidase-peroxidase system and b) the anthrone reaction. Glycogen was extracted in 5% trichloroacetic acid and precipitated with 1.5 volumes of ethanol (CARROLL *et al.* 1956, slightly modified).

Mouse	Glycogen, per cent of wet liver determined by a) enzymes	b) anthrone	a/b, per cent
1	2.93	2.37	124
2	2.18	1.90	115
3	3.46	2.86	121
4	0.88	0.82	107
5	1.41	1.29	109
6	4.44	3.73	119
7	9.01	7.85	115
8	3.70	3.03	122
9	1.94	1.55	125
10	0.97	0.76	128
Mean	3.09	2.62	118

Analysis of variance	Mean square	F	P
Mice	10.029	180.7	<0.001
Methods	1.333	20.4	<0.005
Error	0.0555		

Correlation $= 0.999$ $e_r = 0.01662$ $t = 60.1$ $P < 0.001$

and glycogen was then compared in terms of glucose, which was taken as 100 / (fig. 6)

As can be seen from fig. 6 glycogen and maltose yielded clearly positive reactions in all experiments however the speed of the reaction varied with different enzyme preparations, indicating that it is not just the mere combination of glucose oxidase and peroxidase which is effective, but rather some factor(s) in commercial and purified glucose oxidase which has the ability to split glycogen and maltose

Discussion

Glucose oxidase was discovered in *aspergillus niger* and *penicillium glaucum* by MÜLLER (1928) which was almost completely purified from culture media of *penicillium notatum* by COULTHARD *et al* (1942 & 1945)

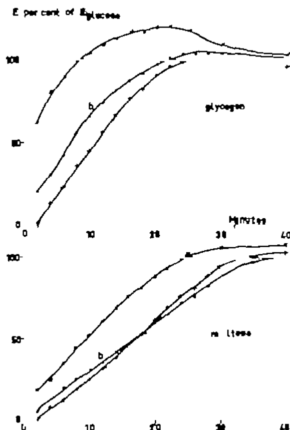


Fig. 6. Comparison of speed f colour development in the coupled enzyme system of glycogen (upper section) and maltose (lower section) using three different glucose xidase preparations. Abscissa: Time in minutes. Ordinate: Extinction relative to glucose, which is taken as 100 % at any measured time. The concentration of all compounds was identical, viz. 0.5 mg/ml.) Reaction with "Fermcozyme" b) Reaction with glucose oxidase "Kabi" c) Reaction with glucose oxidase "Sigma

and extensively studied by KEILIN & HARTREE (1948a, 1948b & 1952) *it is also known as penicillin B or notatin. The latter authors found* that of 50 mono- and disaccharides tested only mannose and xylose were oxidized by the enzyme, though at a rate of about 1 / of glucose. Maltose was practically not oxidized (about 0.2 / of glucose) as measured by oxygen uptake. This finding is not necessarily contradictory to our findings, since KEILIN & HARTREE did not use the coupled enzyme system suggested by KESTON (1956) and introduced by TELLER (1956) for the routine analytical method of glucose determination in blood and urine. HUGGETT & NIXON (1957) and SAIFER & GERSTENFELD (1958) using o-*dianisidin, and MIDDLETON & GRIFFITHS (1957) and MARKS (1959)* using o-tolidin as chromogenic oxygen acceptor emphasized the high specificity

for glucose of the reaction in the coupled enzyme system of commercial purity but do not seem to have performed a more thorough study on specificity

It thus appears from the results mentioned above that the coupled enzyme system glucose oxidase + peroxidase, made from highly purified and now widely used commercial enzyme preparations, has the property of cleaving starch, glycogen and maltose, since it seems to be unlikely that the poly and disaccharide as such would yield a positive reaction It is interesting to note that amongst the poly- and disaccharides tested starch, glycogen and maltose were the only active substances, and that all are built up of 4(alpha-d-glucosido)-alpha-d glucose Whether contaminations in either of the two enzyme preparations or the mere presence of both enzymes together are responsible for the positive reaction with maltose and glycogen is not known at present but the latter suggestion appears to be less likely since coupled systems made up with glucose oxidase from different sources other things being equal, yielded clearly different quotients of E_{max} glycogen/E_{max} glucose as well as E_{max} maltose/E_{max} glucose. In our opinion it is thus most likely that current commercial and research preparations of glucose oxidase contain some factor(s) with rather specific properties capable of splitting 4(alpha-d-glucosido)-glucose linkages and yielding beta-glucose, for which the glucose oxidase reaction is specific (KEILIN & HARTREE 1952) Using the purified stable liquid glucose oxidase preparation "Fermcozyme" the velocity of this assumed cleavage of glycogen must be very high, since peak extinction following addition to the coupled system of glucose was observed only about 90 seconds earlier than following glycogen. In the case of maltose the velocity of the reaction was appreciably lower (fig. 4b) From a practical point of view the findings have yielded a new method for the determination of glycogen, which is more simple and accurate than any known method. As far as is known at present, only starch, glycogen, maltose, and glucose react in the system, and if the latter two are suspected of being present in a sample in measurable amounts, they may be readily eliminated by 30 minutes of incubation at pH 12 and 95° without any loss of glycogen. Since acid hydrolysis of glycogen did not give total recovery it is assumed that the method mentioned above will be more accurate than those involving acid hydrolysis and subsequent sugar determination The sensitivity of the method as adopted here is of the same order for glycogen and glucose (lower limit = about 5 µg) which is more than sufficient for liver glycogen determinations. It can, however be increased if necessary

Added in proof After obtaining lyophilized glucose oxidase of the highest degree of purity (130 U/mg) it has now been confirmed that this enzyme does *not* convert glycogen, but factor(s) present even in relatively highly purified preparations, most likely highly active amylase.

Summary

It was observed that the so-called specific method of glucose determination by means of current glucose oxidase preparations yields quantitative reactions with glycogen, starch, and maltose. Two preparations of highly purified glycogen from different sources showed agreement with Beer's law and equal potency. On acid hydrolysis of glycogen, the glucose oxidase positive material first decreased to about half the initial value and subsequently increased at a slower rate to about 85 % of the initial reading. The method of GOOD *et al.* (1933) is thus not suitable to replace the copper reducing reaction by the glucose oxidase system, since the activity measured in the latter fluctuates with time, yielding unsatisfactory recovery.

Dissolving purified glycogen or mouse liver glycogen precipitate at pH 12.5 and 95° followed 30 minutes later by analysis in the coupled enzyme system, proved to be an accurate method for liver glycogen determinations, which is specific against maltose and glucose. The method outline above has been compared with known methods (copper reduction following acid hydrolysis or anthrone reaction) and the results are discussed. The new method of glycogen determination is far more rapid, convenient, and accurate than the hitherto known methods. The sensitivity is the same as for the determination of glucose. The speed of colour development in the coupled enzyme system using o-tolidin as chromogenic oxygen acceptor is highest for glucose (9 minutes at 23°), slightly lower for glycogen (10.5 minutes), and slowest for maltose (16 minutes) suggesting that glycogen and maltose do not react as such but after cleavage to glucose by some yet unknown mechanism.

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The Effect of Ethanol on the Membrane Permeability to Sodium and Potassium Ions in Frog Muscle Fibres

By

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(Received May 26, 1966)

In a previous investigation ethanol was shown to depolarize the membrane of frog muscle fibre and simultaneously reduce the membrane resistance it was suggested that ethanol might increase the membrane permeability to one or more ions (KNUTSSON 1961) Investigations performed on squid axon have shown that ethanol in high concentrations reduces the ionic permeability changes occurring during activation, the membrane permeability to sodium being more affected than the potassium permeability (MOORE 1958 ARMSTRONG & BINSTOCK 1964) In heart muscle fibre ethanol also seems to act selectively on the permeability to different ions during activation, since the rate of repolarization of the action potential is markedly increased even at low concentrations of ethanol, whereas the depolarization rate and the amplitude of the action potential are not significantly altered (GIMENO, GIMENO & WEBB 1962) The question then arises whether ethanol also has selective effects on the permeability of the resting membrane to different ions.

The present investigation was undertaken to determine selective effects of ethanol on the resting membrane permeability to sodium and potassium ions in frog skeletal muscle fibre. The ethanol concentration used was 50 mM which is the lowest shown to depolarize the membrane of these fibres. The experimental design was based on the assumption that the resting potential of muscle fibre arises as a consequence of the distribution of ions across the membrane as well as of the permeability of the membrane to these ions. The effects of ethanol on the permeability of the membrane were studied by measuring the effective resistance and membrane potential changes in muscle fibres in external fluids of different ionic compositions.

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Methods

Isolated sartorius muscles of frog (*Rana temporaria*) were used which when dissected were placed in bath with the deep surface pointing upwards. In most f the experiments the muscle was kept without any fixation at the bottom of the 4 ml bath, the composition of which was changed as described under Results. The temperature of the bath was kept at 14 by circulating water from thermostatically controlled bath through a container surrounding the muscle bath.

The resting membrane potential was measured by internal capillary lectrodes with tip diameters f less than 1 μ and resistance between 10 and 20 megohms. They were filled with 2.7 M potassium chloride and had tip junction potentials (ADRIAN 1956) f less than 5 mV The changes in tip junction potential when altering the ionic composition of the muscle bath were 2–6 mV the addition of ethanol to any of the bath fluids changed the tip junction potential by less than 1 mV A membrane potential change observed after alteratio f the ionic composition of the bath may thus have been somewhat influenced by a change i the tip j nction potential, while the membrane potential change on ddition of ethanol t the bath was not significantly affected by alterations of potentials at the tip junctio Routinely the membrane potential was measured i 12 randomly chosen fibres, the t me necessary for these measurements being as a rule 2 3 minutes. With the muscle in Ringer's fluid, resting potentials with mean value of 88–90 mV and standard deviation (S.D) of less tha 2 mV were considered to indicate satisfactory conditions for both electrode and muscle fibres. After each change of the bath fluid the muscle was left to soak for period of bout 10 minutes before new measurements were made. The selection f the 10-m nute interval was based on the observation that during the first few minutes after changing some of the solutions the muscle fibres twitched spontaneously or went into contracture. I one series of experiments the membrane potential was recorded continuously for 10–15 minutes and ethanol added to the bath during the recording (cf. KNUTSSON 1961).

Changes i the effective resistance of the muscle fibre (cf FATT & KATZ 1951) induced by ethanol were determined by recording the voltage-current relation across the muscle fibre membrane. Two electrodes were inserted into the same fibre at an inter-electrode distance f approximately 30 μ. One electrode was used to pass electrical current across the fibre membrane and the other to record the resultant potential change. Continuously rising current pulses were used the rat of increase of the current being maintained at bout 0.1 μA/sec. This rate is sufficiently slow to exclude any significant influence of the membrane capacitance on the polarization induced by the current (KNUTSSON 1964). The current passing the stimulating electrode was recorded as a RI drop across 10,000 Ω resistor i series with the current supply The X Y display feature of the oscilloscope was used, and variation of applied current i tensity recorded as horizontal deflexion and concomitant changes in membrane potential as vertical deflexion of the oscilloscope beam. Thus direct recordings of voltage-current curves were obtained (cf. HUTTER & PADSHA 1959). The voltage-current ratio was determined at 5-minute intervals until series of determinations showed equal voltage-current curves. Ethanol was then added and recordings made gain at 5-min te intervals.

Solutions. Solution 1 in table 1 is Ringer's fluid, lik that used by ADRIAN (1956). Solution 2 was obtained by add g small vol me of NaCl solutio to the muscle bath containing solution 1 Na-free Ringer' fluid (solution 3) was made by replacing N with choline on a mole-for-mole basis, and Na-free solution with high Cl concentration (sol tion 4) by addition of a choline chloride solution to the muscle bath containing solution 3 The toxicity of solutions 2 nd 4 was pproximately twice that of Ringer's fluid So

Table 1

Composition of solutions.

Solution	K	Cl	Na	Ca^{2+}	HPO_4^{2-}	$H_2PO_4^-$	SO_4^{2-}	$Choline^+$	Sucrose (m-mole/l solution)	Relative toxicity
	mg ion/l solution									
1	2.5	121	120	1.8	2.15	0.85	–	–	–	1
2	2.5	241	240	1.8	2.15	0.85	–	–	–	2
3	2.5	118.6	–	1.8	1.04	0.42	–	115	–	1
4	2.5	238.6	–	1.8	1.04	0.42	–	235		2
5	80	–	–	8.0	1.08	0.43	45	–	115	1

5 was Na- and Cl-free solution containing 80 mg ions/l of potassium. Isotonicity was maintained by the addition of sucrose. This solution had a concentration of 8 mg ions/l of Ca, which was the highest concentration that could be taken up into solution. The ionized calcium was then only about half of that in the Ringer's fluid (cf. Bauer 1954). Routinely the ethanol concentration was 50 mM (2.3 g/l). Only in one series of experiments in which no effect was seen at this concentration were the higher concentrations of ethanol, i.e. 100–500 mM, used.

Results

Ethanol effects on the membrane permeability to sodium ions

Figure 1 shows the effect of ethanol (50 mM) on the membrane potential of frog muscle fibres in Ringer's fluid (solution 1 table 1). In the three experiments illustrated, the mean membrane potential decreased by 7 5 and 2 mV respectively. These results are in good agreement with

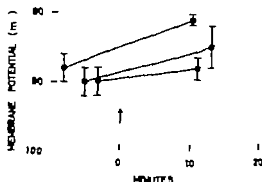


Fig. 1 Effect of 50 mM ethanol on resting potential of frog muscle fibres in Ringer's fluid. Resting membrane potential from three experiments before and after addition of ethanol (arrow) to the bath. Vertical lines refer to two standard deviations and each point is the mean of 12 measurements.

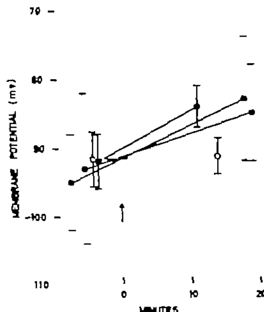


Fig. 2. Effects of 50 mM ethanol on membrane potential of fibres in Na-rich hypertonic solution. *Filled circles* represent mean membrane potentials (vertical bars 2 × S.D.) from three experiments: values *to the left* of the arrow obtained 10 minutes after increasing the external NaCl concentration to about twice the normal; values *to the right* of the arrow obtained after addition of ethanol at arrow. *Open circles*, control experiment without addition of ethanol.

previous observations made during continuous recordings of the membrane potential in single fibres exposed to ethanol in the same concentration (KNUTSSON 1961). By contrast, fibres in Na free Ringer's fluid in which the Na had been replaced by choline were not depolarized by ethanol. Thus, *in experiments performed on muscles equilibrated for* 30 minutes in Na-free medium (solution 3) the mean membrane potential in two cases was found to be unchanged 10–15 minutes after addition of ethanol, and in a third case there was only an insignificant decrease of 2 mV. These results support the view that ethanol increases the membrane permeability to sodium. Thus, with the muscle in normal Ringer's solution there would be an increased inflow of sodium, resulting in a shift of the membrane potential in a positive direction, whereas in the absence of external sodium ions such a depolarization could not take place. From a theoretical point of view the addition of ethanol should result in a more marked depolarization after an increase of the sodium concentration in the external solution (cf. SHANES 1958): that this is actually the case is shown by the experiments on fibres in sodium-rich Ringer's fluid (solution 2) described below.

In these experiments the resting potential was first measured in normal Ringer's solution, in order to test the condition of the fibres and the accuracy of the recordings (see Methods). If these were found to be satisfactory, the NaCl concentration in the bath fluid was increased by adding a pre-determined amount of a concentrated NaCl solution. After allowing ten minutes for equilibration, the resting potential was measured in 12 fibres: the mean values (and standard deviations) obtained in three experiments are indicated by the filled circles to the left of the arrow in fig. 2. The mean membrane potentials thus obtained were −92 to −95 mV, i.e. somewhat higher than normal, in agreement with previous results of experiments with increased external NaCl concentration (NASTUK & HODGKIN 1950, cf. below). When the membrane potentials had thus been stabilized, ethanol was added (arrow) up to a concentration of 50 mM, and 10–20 minutes later the mean values of the membrane potential had decreased by about 10 mV, whereas in the control experiments (open circles) performed in the same way except that ethanol was not added, the potential remained unchanged over a corresponding period of time.

The results described represent membrane potential changes measured in different fibres chosen at random (cf. Methods). Although technically more difficult, a comparative series of nine experiments was also performed, in which the membrane potential changes induced by ethanol were continuously recorded in one and the same fibre. Since after the addition of NaCl the fibres usually twitched spontaneously during the first few minutes, causing either dislodging or breaking of the electrode, the measurements were not started until 5–7 minutes after increasing the NaCl concentration. After a further 5-minute interval ethanol was added to the bath in the course of the continuous recording. The result as measured 10 minutes after the ethanol addition was invariably a depolarization, ranging from 6 to 12 mV, whereas in the corresponding control experiments, following the same procedure except that ethanol was not added, the depolarization ranged from 0 to 6 mV.

The two series of experiments clearly show that the depolarizing effect of ethanol is much more marked and regular in fibres in a sodium-rich solution than in Ringer's fluid. However, since in these experiments the tonicity of the external fluid after the addition of NaCl to the bath was twice that of the normal Ringer's fluid, water can be expected to be withdrawn from the fibre. This may increase the internal potassium concentration, resulting in a raised concentration gradient for potassium across the membrane, which may explain the hyperpolarization observed after equilibration in this medium. It was thus considered that two complementary series of comparative experiments should be performed using

Na-free solutions of the same high tonicity In one series, choline chloride was added to the muscle bath in an amount giving virtually the same external chloride concentration and tonicity as in the experiments shown in fig. 2. In the other series, the tonicity was increased to twice the normal by the addition of sucrose to the bath. In both these types of experiments the resting potential increased to about −110 mV after equilibration in the hypertonic solution, in agreement with the findings described above. During the period of action of ethanol the resting potential remained virtually unchanged at this hyperpolarized level in six experiments, as measured 10 minutes after the addition of ethanol (50 mM)

As is evident from these results there is an essential difference between the effects of ethanol in the presence and in the absence of external sodium ions. Whereas in the presence of external sodium ions ethanol causes a lowering of the membrane potential, it has no significant effect when the external sodium has been replaced by choline, which lends support to the assumption that ethanol increases the membrane permeability to sodium ions.

The effect of ethanol on the membrane permeability to potassium ions

According to Conway & Moore (1945) the frog muscle fibre membrane is impermeable to sulphate ions. In fibres placed in a potassium sulphate solution, currents passing across the membrane should thus chiefly be carried by potassium ions Consequently it might be possible to establish the effect of ethanol on the membrane permeability to potassium, by observing the effects of ethanol on the membrane conductance in fibres in a potassium sulphate solution.

For the present series of experiments, a solution containing 80 mg ions/l of potassium was used This was the highest concentration at which definite and stable negative resting potentials could readily be obtained, thus ensuring that the electrodes had penetrated the membrane. In practice, the resting potentials ranged between −8 and −16 mV thus agreeing reasonably well with the potassium equilibrium potential which was calculated to be −14 mV on the assumption that the internal potassium concentration is 140 mg ions/l (Adrian 1956).

The effect of ethanol on the membrane conductance was studied by determining the voltage-current ratio across the membrane of fibres before and during exposure to ethanol. Direct voltage-current curves were obtained by applying slowly continuously rising currents and recording the voltage change across the membrane as a function of the applied current. In such a curve, each point gives the voltage-current ratio for a particular current amplitude and hence the curve represents a large

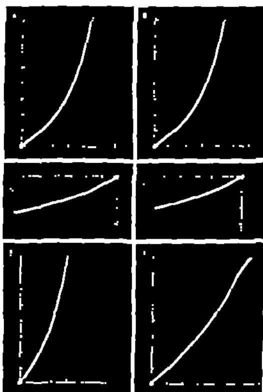


Fig. 3 Voltage-current curves from muscle fibre in potassium sulphate solution during linearly rising outward (*A*) and inward (*C*) current. *B* and *D* the corresponding curves 5 minutes after addition of ethanol (50 mM) to the bath. *E*, voltage-current curve from another fibre in potassium sulphate solution during outward current, and *F* the corresponding curve 5 minutes after addition of 500 mM ethanol to the bath. Resting potential at intersection of vertical voltage axis and horizontal current axis −14 mV in both fibres. Scale divisions on voltage axis 10 mV on current axis 0.05 μA. Interelectrode distance 30 μ.

number of determinations at different current strengths (FURSHPAN & POTTER 1959) This type of curve was considered to be suitable since the increase in voltage with the current applied across the membrane of fibres in potassium sulphate solution is non-linear (KATZ 1949) which may be due to variations in membrane permeability to potassium ions with the membrane potential level (cf HODGKIN & HOROWICZ 1959).

A typical experiment is illustrated in fig. 3 Records *A* and *C* show the voltage-current curve during outward and inward current, respectively and *B* and *D* the corresponding curves 5 minutes after the addition of ethanol in an amount sufficient to give a concentration of 50 mM. The records show that ethanol did not produce any change in the slope of the curves, thus indicating that the effective resistance of the fibre had not undergone any change large enough to be detected by the method

used. We estimate that we could have seen a change of 5 or 10 / The same result was obtained in all six fibres tested, independently of whether the tests were made 5 or 10 minutes after the addition of ethanol. The results indicate that the permeability of the membrane to potassium ions is not appreciably affected by ethanol in this concentration. Nor had ethanol any effect on the voltage-current curve in concentrations of 100, 200 or 300 mM. Only at a concentration as high as 500 mM did ethanol cause a change, as illustrated in fig. 3*E* and *F* which show the voltage current curves during outward current pulses before (*E*) and after (*F*) the addition of ethanol. In this case, the slope of the curve is considerably less steep after the addition of ethanol, indicating a decrease in the effective resistance of the fibre. However no detailed analysis was made of the effects of ethanol on the membrane permeability at this high concentration.

Discussion

The results obtained in the present investigation indicate that ethanol at a concentration of 50 mM increases the permeability of frog muscle fibre membrane to sodium ions but has no effect on the membrane permeability to potassium, considerably higher concentrations being required to obtain changes in membrane conductance which may be attributable to an altered potassium permeability Thus, it appears that the sodium permeability of the membrane is more affected by ethanol than is the potassium permeability It cannot be excluded, however that the difference thus observed may to some extent be due to the fact that different methods were used for the determinations of the effects of ethanol on the membrane permeabilities to the two ions. Whereas in the studies on the potassium permeability the change in effective resistance of fibres immersed in potassium sulphate solution was determined, the effects on the sodium permeability were deduced from changes in the resting potential of fibres in the presence and in the absence of external sodium ions. This latter method was chosen because the sodium conductance constitutes only a small part of the total membrane conductance and hence it was considered impracticable to identify the effects of ethanol on the sodium permeability by determining conductance changes across the membrane. Different methods were tried for determining the effects of ethanol on the potassium permeability by some method analogous to that used to study effects on the sodium permeability but none of them gave unequivocal results. Thus it seems impossible at present to determine exactly the relation between sodium and potassium permeability changes during the action of ethanol.

Previous investigations on the effects of ethanol on the ionic permeabilities of cell membranes have primarily been concerned with the permeability changes during voltage clamp. They have established that ethanol depresses the maximum membrane conductance for sodium and potassium (MOORE 1958, ARMSTRONG & BINSTOCK 1964, MOORE, ULBRICHT & TAKATA 1964) and have shown that the sodium conductance is far more reduced than is the potassium conductance, the only exception being the results reported by MOORE *et al.* (1964) using the sucrose-gap technique, from which it appeared that the presence of sucrose increases the susceptibility of the potassium conductivity to ethanol. Thus, there is well-founded evidence that the sodium conductance is much more sensitive to ethanol than is the potassium conductance, not only in squid axon during activation but also in frog muscle fibre during rest, as indicated by the present investigation.

The previously observed increase in the total membrane conductance of muscle fibres exposed to ethanol (KNUTSSON 1961) may in part be attributable to the increase in membrane permeability to sodium ions revealed in the present investigation, but may of course also be due to changes in the membrane permeability to other ions. From the results obtained in this investigation it is reasonable to conclude that the potassium permeability is not appreciably affected by ethanol in low concentrations, since concentrations up to 300 mM did not affect the effective resistance of fibres in potassium sulphate solution. As far as the effects of ethanol on the permeability to other ions are concerned, only the chloride permeability has so far been studied. These studies (to be published separately) have not yet yielded fully consistent results but suggest that the membrane permeability to chloride is also increased by ethanol. On this assumption, the slow and irregular depolarizations of the muscle fibre membrane exposed to ethanol in low concentrations should be due to its combined effects on the sodium and chloride permeabilities. Since an increase in the sodium permeability would move the membrane potential toward the sodium equilibrium potential, it would tend to depolarize the fibre membrane, whereas an increase in the membrane permeability to chloride would stabilize the membrane potential, since the equilibrium potential for chloride is equal to or close to the resting potential.

Summary

An investigation was made of the effects of ethanol on the membrane permeability to sodium and potassium in isolated sartorius muscles of the frog.

Sodium permeability changes were studied by recording the resting membrane potential in the presence and in the absence of external sodium ions, using intracellular capillary electrodes. Effects on the potassium permeability were studied by determining the change in effective resistance of fibres immersed in a potassium sulphate solution

Ethanol in a concentration of 50 mM caused only a slight membrane depolarization of fibres in normal Ringer's fluid but markedly decreased the resting potential of fibres in Na rich solutions, the mean resting potentials in randomly chosen fibres being reduced by about 10 mV whereas in continuous recordings from one and the same fibre a depolarization ranging from 6 to 12 mV was observed. The resting potential of fibres in solutions in which the sodium had been replaced by choline was unaffected by ethanol. It was concluded that ethanol might increase the sodium permeability of the membrane.

The effective resistance of fibres immersed in potassium sulphate solution was unaffected by ethanol in concentrations of 50 up to 300 mM but decreased at a concentration of 500 mM. The results thus support the view that ethanol in the lower concentrations used has no effect on the membrane permeability to potassium since the currents across the membrane of fibres in potassium sulphate solution are mainly carried by potassium ions.

Acknowledgements

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Effects on Pregnancy and Offspring in Rats and Mice of Bis (P Acetoxyphenyl) Cyclohexylidenemethane (F6066)

By

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(Received May 27 1966)

Bis (p-acetoxyphenyl) cyclohexylidenemethane, F6066 is a weak oestrogenic substance with a marked effect on the reproductive system. The substance (fig. 1) is one of a series of diphenylethenes synthetised by *inter alia* MIQUEL, WÅHLSTAM, OLSSON & SUNDBECK (1963).

The substance proved to be non-toxic in mice, when given in solution in oil by mouth for 2 days in a dose of 1600 mg/kg per day or in aqueous suspension in a dose of 125 mg/kg per day for 3 months in rats.

F6066 has a weak uterotropic effect in mice and a weak vaginotropic effect in ovariectomised rats. F6066 reduces the weight of the testes and ventral prostate in male rats (pituitary inhibition test) and is a more potent hypophyseal inhibitor in relation to its oestrogenic effect than is oestradiol and stilboestrol. F6066 has an anti-gestagenic effect in rats and rabbits. A reversible sterility can be demonstrated during and some weeks after the administration of F6066 to male and female rats (EINER JENSEN 1965).

F6066 has no demonstrable toxic effect on dogs when given in a single dose of 1000 mg/kg in gelatin capsules or up to 200 mg/kg a day in the form of tablets for 3 months (unpublished data from our laboratory).

Using autoradiographic examination of whole body distribution of ^{14}C F6066 in mice HANNGREN, ULLBERG & EINER JENSEN (1965a & b) demonstrated a specific, very marked accumulation of the substance in active corpora lutea. Radioactivity was also demonstrated in the pituitary adrenal cortex, endometrium, the interstitial tissue in the testicles and ovaries and in the liver and bile.

LARSSON (1966) found that F6066 is excreted in the faeces and urine partly as deacetylated and conjugated glucuronic acid and partly in the form of very polar metabolites. Even after 4 days the urine was still fairly radioactive.

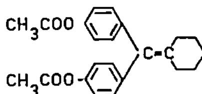


Fig. 1 F6066.

NYLANDER & TERNER (1964) showed that F6066 has a favourable effect in the treatment of prostatic cancer PERSSON (1965a) found changes in the excretion of gonadotropin and a marked reduction of the oestradiol 17β-excretion in the urine. These findings prompted PERSSON (1965b) to use F6066 in an attempt to stimulate ovulation in women with a disturbance of the normal pituitary-ovarian inter relation.

The purpose of the present investigation was to ascertain whether F6066 has any substantial teratogenic effect in animals which might contraindicate its use in the treatment of women in the child-bearing age

Method

NMRI-mice purchased from commercial breeder (Friis, Laven, Denmark) and rats from our own what 15-year old closed colony were used. All the female animals were virgin but males known t be fertile were used. During the pairing period the animals were kept in plastic cages, 1 male and 2–4 females in each cage. Every morning at 8 m. vaginal smears were taken from the female rats for microscopic examination for spermatozoa. In mice the presence of sperm plug in the vagina was taken as evidence that coitus had occurred. I rder to avoid the necessity f killing animals on Saturdays and Sundays, some animals which, calculated from the day of conception, should have been killed on these days were moved to our general breeding house. In view of this and the lack of evidence of conception in some of the experimental animals, the number of females used was 30–100% more than the actual number required. But even then it was occasionally necessary to kill the animals 1– days before th pre-determined day These embryos were not included in the calculation f the erage weight f the foetuses, since their weight increases by almost 100 / on the last day of intra-uterine life.

The pregnant animals were randomly distributed am ng experimental groups from day to day It took up to 4 weeks to collect sufficiently large groups of mice, and 1 2 weeks for rats.

The animals were kept in rooms with d ylight and artificial light from 6 a.m. until midnight during the mating period, and in room with only daylight during pregnancy The room temperature was about 23 and the animals were allowed pellets and w ter *ad lib*

Treatment days were determined in relation to the day when spermatozoa were demonstrated or vaginal plug was found (called d y 0) The substance was generally given

for 2 or 3 days. F6066, dissolved in 96 % olive oil and 4 % absolute alcohol was administered by stomach tube. Trypan blue*) was used as positive control and was given subcutaneously in 0.9 % NaCl (BECK & LLOYD 1964). Olive oil with 4 % absolute alcohol was used as a negative control.

The female rats were killed on day 20 and the mice on day 18 (a few animals 1–2 days earlier). The mothers were anaesthetised with ether and bled. The uterus was opened and the number of living and dead foetuses was noted as well as the lengths in millimetres of the dead foetuses. Large foetuses were freed from the membranes, after which they were weighed and examined under a Leitz stereomicroscope (magnification ×10). In the last mentioned experiments the ano-genital distance was measured with a measuring ocular. The abdominal and intrathoracic organs were then removed and examined under the stereomicroscope.

The foetuses were fixed in 80 % ethyl alcohol. They were then placed in 1.0 % NaOH solution for 3 days and every day they were rinsed with water and placed in a fresh solution. For small foetuses 0.5 % NaOH was used. The bodies were then stained for 24 hours at room temperature with a mixture of 3 ml 0.1 % alizarin dissolved in 1.0 % NaOH + 100 ml 0.5 % NaOH (the same amount of staining fluid per animal). The animals were then rinsed in water and placed in 20 % glycerine. When the embryos had sunk to the bottom they were transferred to 50 % glycerine and then to 100 %. (Modified according to BROCK & VON KREYBIG 1964 and LORKE 1963.)

The animals can be kept in concentrated glycerine. The bodies of the animals were now clear and translucent and the skeleton was bright red-blue in colour. The skeleton was examined under the stereomicroscope for skeletal abnormalities. The ribs, sternal bones and vertebral bodies were also counted.

In one experiment (R4) the female rats were allowed to give birth and keep their young. The mothers were given either olive oil or F6066 dissolved in olive oil by stomach tube from the 7th day of pregnancy until 5 days after birth. On day 5 some of the young were killed and the testicles, ventral prostate, seminal vesicles or ovaries and uterus were removed and photographed so as to compare the sizes of these organs in the various groups. Some of the newborns were killed when weaned and the sex organs weighed. One male and 1 female from each litter were paired at 3 months of age with offspring from another mother in the same group in order to check whether the animals were fertile. Only half of the surviving control animals were paired.

The days on which the animals were treated with F6066 and the doses used are given in tables 1, 2 and 3.

Results

Rats

A dose of 8 mg/kg of F6066 or less a day on days 7–8–9 after mating produced no reduction in the number of living foetuses in rats (table 1 R 1, R 2). When the mothers were given 16 mg/kg a day on days 7–8–9 or 7–8 the number of foetuses was normal (R 2) or reduced (R 5) and when the daily dose given was 32 mg/kg, the number was reduced.

Administration of F6066 in a dose of 64 mg/kg a day from day 7 until 5 days after birth, resulted in only 3 of 10 females giving birth to

*) Trypan blau, Standard Fluka, Fluka AG Switzerland

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Table 1

Antifertility and teratogenic tests with F6066 on rats.

Test	Substance adm. by stom.tube day	Dose/day mg/kg	Substance	Numb. of pregn/ numb. of rats	Young born before killed	Total numb. of liv foetuses per group ♀	Total numb. of liv foetuses per group ♂	Average weight of living foet. killed on day 20	Total number of dead foetuses per group > 3 mm	Total number of dead foetuses per group < 3 mm	Dead foet. per pregn rat	Rectogenital distance mm ♀	Rectogenital distance mm ♂
R 1	7–8–9	1 ml/kg	Olive oil	6/6		30	28	3.19	1	9	1.6		
		2	F6066	5/6		18	31	3.24	–	5	1.0		
		4	F6066	4/6		25	16	3.88	1	1	0.5		
		8	F6066	5/5		23	29	3.24	1	7	1.6		
R 2	7–8–9	1 ml/kg	Olive oil	9/10		56	43	3.11	1	6	0.8		
		8	F6066	9/10		36	49	3.13	–	12	1.3		
		16	F6066	10/10		46	51	3.27	–	9	0.9		
R 3	1 2	1 ml/kg	Olive oil	5/5		23	25	2.97	1	2	0.6		
	1 2	32	F6066	1/5			–	–1)	1	–	1.0		
	3–4	32	F6066	3/5		19	7						
	5–6	32	F6066	5/5		17	18	2.44	1	9	2.0		
	7–8	32	F6066	3/5		14	12	3.22	1	5	2.0		
R 5	1 2	2 ml/kg	Olive oil	7/8		33	48	3.39	1	4	0.7	3.0	4.6
	1 2	16	F6066	0/8		–	–	–	–	–	–	–	–
	3–4	16	F6066	7/8		17	21	2.95	11	14	3.6	2.9	4.5
	5–6	16	F6066	5/8		19	20	3.34	5	2	1.4	2.9	4.8
	7–8	16	F6066	6/8	9	25	23	3.21	3	2	1.0	3.0	4.6
R 6	7–8–9 sc	2 ml/kg	0.9 % NaCl	8/8	18	29	27	3.19	1	4	0.6	2.9	4.5
	7–8–9 sc	25	Trypan blue	5/8		19	27	3.23	1	6	1.4	2.9	4.4
	7–8–9 sc	50	Trypan blue	4/8	8	17	11	3.30	–	–	–	2.7	4.5
	7–8–9 sc	100	Trypan blue	2/8		1	1	2.55	11²)	2	6.5	2.5	–

1) 8 small, but living young (a weight 0.69 g) in one rat are not included in this figure.
2) foetuses from one mother

Table 2

Antifertility and teratogenic tests with F6066 on mice.

Test	Substance adm. by stom. tube day	Dose/day mg/kg	Substance	Numb. of pregn / numb. of mice	Young born before killed	Total numb. of liv foetuses per group ♀	Total numb. of liv foetuses per group ♂	Average weight of living foet. killed day 18	Total number of dead foetuses per group >3 mm	Total number of dead foetuses per group <3 mm	Dead foet. per pregnancy	Rectogenital distance mm ♀	Rectogenital distance mm ♂
M 1	6–7–8	10 mg/kg	Olive oil	6/10	9	21	32	1.34	–	6	1.0		
	6–7–8	32	F6066	1/10		4	5	–	–	–	–		
	6–7–8	64	F6066	2/10		7	9	1.25	–	–	–		
	6–7–8 sc	100	Trypan blue	6/10	8	18	27	1.29	1	10	1.8		
M 2	1	10 mg/kg	Olive oil	1/4		4	2	1 17	–	–	–		
	1–2	64	F6066	1/5	–	1	0	0.75	–	–	–		
	3–4	64	F6066	0/5				–	–	–	–		
	5–6	64	F6066	0/5		–	–	–	–	–	–		
	7–8	64	F6066	2/5	–	7	8	1.22	–	2	1.0		
M 3	1 2	10 mg/kg	Olive oil	5/6		24	22	2.8	1	3	0.8	2.1	3 1
	1 2	64	F6066	1/6	–	–	–	–	2	3	5.0	–	–
	3–4	64	F6066	0/6	–	–		3–	–	–		–	–
	5–6	64	F6066	0/6		–		1 7	–	–	–	–	
	7–8	64	F6066	2/6	5	0	2	11	3	–	5.0	–	3.3
M 4	6–7–8	10 mg/kg	Olive oil	6/8		20	33	1 7	–	8	1.3	2.1	3.0
	6–7–8	8	F6066	3/9		4	4	1 41	4	3	2.3	2.0	3.1
	6–7–8	16	F6066	5/8	–	19	20	1.22	2	10	2.4	2.0	3 1
	6–7–8	32	F6066	2/9		5	4	1 19	2	3	2.5	2.1	3 0
M 5	1 2	10 mg/kg	Olive oil	5/8	–	18	16	1.34	3	6	1.8	2.2	3.2
	1 2	16	F6066	1/8	–	2	3	0.63	–	2	2.0	1 8	2.5
	3–4	16	F6066	0/8	–	–		–	–	–	–	–	–
	5–6	16	F6066	2/8	–	5	8	1.23	5	1	3.0	2.3	3.1
	7–8	16	F6066	5/8	–	16	17	1.23	2	9	2.2	–0	3 0

Table 3

Antifertile nd abortive effects of F6066 given to pregnant rats by stomach tube from day 7 of frequancy to day 5 after the birth.
Test R 4

Animal No.	F6066	Number of young F		Numb. of ♀ and ♂ verage weight of animals killed on day 5 after birth						Average weight of organs after weaning, mg					Number of young F_2 after remaining F_1♀		Average weight at birth
		born	5 days later	♀	♂	weight g	♀	♂	weight g	ovaries	uterus	testes	pro-stat	semves	born		
1 1	16 mg/kg	9	8	2	1	9.0	2	1	37	14	18	210	23	8.0	8	The test was discontinued 46 days after the males and females were placed together	6.6 g
1 2				–	–	–	–	–	–	–	–	–	–	–			
1 3		9	9	2	2	8.0	–	–	–	–	–	–	–	–			
1 4		11	11	2	3	7 4	2	2	39	15	23	169	23	8.0	progn.		
1 5		–	–	–	–		–	–	–	–	–	–	–	–			
1 6		–	–	–	–	–	–	–	–	–	–	–		–			
1 7			–	–	–	–	–	–	–	–	–	–	–	–			
1 8		13	13	2	3	10.2	–	2	43	–	–	183	19	6.5	12		5.1 g
1 9	"	1 (dead)	–	–	–	–	–	–	–	–	–	–	–	–			
1 10		8	8	–	3	10.7	1	2	43	17	22	179	24	8.0	10		4.8 g

II 1	64 mg/kg			–	–		–	–	–	–		–		–	7	7.7 g
II 2		3(+1 dead)	3	1	–	13.0	–	–			–	–	–	–		
II 3				–	–		–	–			–	–	–	–		
II 4			–	–	–		–	–	–		–	–	–			
II 5		–		–	–		–	–	–			–	–			
II 6				–	–		–	–	–	–	–	–	–	–		
II 7				–	–		–	–		–			–	–		
II 8		1(+3 dead)		–	–	–	–	–				–	–			
II 9		1 (dead)		–	–	–	–	–				–	–	–		
II 10		–		–	–		–	–	–	–	–	–	–	–		
III 1	Olive oil 1 ml/kg	12	12	2	3	8.4	1	–	48	27	32	–	–	–		
III 2		13	13	3	3	7.7	3	2	40	18	24	185	25	6.5	8	6.4 g
III 3		6	6	1	1	12.0	1	1	36	13	23	143	17	9.0	pregn.	
III 4		10	10	2	2	7.3	2	2	35	13	20	154	23	8.5	7	7.0 g
III 5		9	9	3	1	8.0	2	–	41	21	27	–	–		not pregn.	
III 6		9	9	–	3	13.7	2	2	44	19	25	170	20	7.0		
III 7		10	10	1	3	8.5	2	3	43	17	20	198	23	7.0		
III 8		5	5	–	2	13.0	1	2	45	15	27	209	21	7.5		
III 9		7	7	2	1	9.0	1	1	40	14	22	170	19	8.0		
III 10				–	–	–	–	–		–	–	–	–			

living offspring and on day 5 only 3 newborns from one litter were still alive (table 3) Similar treatment of 10 female animals but with 16 mg/kg resulted in the birth of 6 litters and in these, only 1 of the animals in one of the litters was found dead. The growth of these newborns, the appearance of their sexual organs of those killed on day 5 and the weight of the sexual organs of animals killed when weaned were the same as in the control group The fertility of the animals at 3 months was also the same as in the control animals.

Mice

In these animals the number of living foetuses of mothers treated with 8–64 mg/kg F6066 a day on days 6–7–8 (M 1 and M 2 in table 2) was reduced. Sixteen or 64 mg/kg injected on days 1–2, 3–4 or 5–6 resulted in impaired implantation or total abortion in most of the mothers, while the effect of F6066 diminished rapidly when given after implantation (days 7–8) This is clear from M 3 and possibly from M 2. M 3 is a repetition of M 2 and was performed, because the lack of fertility in the control group in M 2 made it difficult to draw any conclusions on the fertility of the groups that received the substance.

On 3 occasions (R 3 M 2, R 5) we found that foetuses from mothers treated with F6066 were substantially smaller than those of control foetuses. The foetuses were living and normally developed as far as we could see with our technique. Eight rat foetuses with an everage weight of 0 69 g compared with normally more than 3 g, were found in the same mother in R 3 The 8 foetuses were examined at the Embryological Institute in Lund by Professor B Källén, who found them somewhat premature, but showing no signs of malformation Examination of the

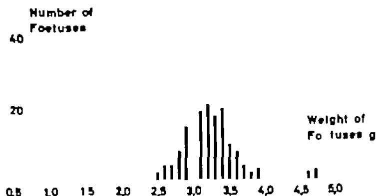


Fig. 2. Weight distribution of 178 rat foetuses on day 20 from pregnant control rats. The 11 foetuses weighing 4.0–4.9 grams are from the same mother

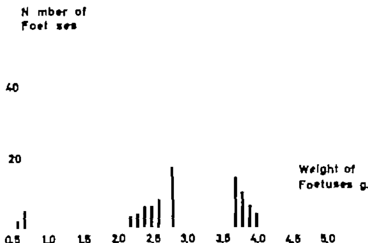


Fig. 3 Weight distribution f 392 rat foetuses on day 20 from rats given 2 32 mg/kg F6066 during 2 or 3 days of pregnancy The 8 foetuses weighing 0.6–0 8 grams are from the same mother The type of distribution is the same as in fig. 4.

6 small mouse foetuses (tabel 2, M 2, M 5) after careful staining with alizarin showed normal maturation and no skeletal changes. Apart from the low weight of these few animals, no difference in mean weight and weight distribution (fig 2, 3 and 4) was found between the F6066- and control groups.

In both the mice and the rats F6066 increased the number of dead foetuses per pregnant mother (tables 1 and 2). Tables 1 and 2 give the number of pregnant animals per group *i.e* the number of animals found to contain living or dead foetuses when killed.

Some of the "not pregnant" animals had been pregnant at one stage since the uterine wall was nodular in some areas, *e.g* in 5 of the 6 mice that had received 8 mg/kg bodyweight on days 6–7–8 in M 4 and did

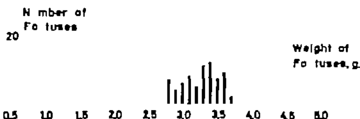


Fig. 4. Weight distribution of 76 rat foetuses on day 20 from rats given 25–100 mg/kg Trypan blue on day 7–8–9 of pregnancy

Table 4

Type nd number of major malformations of living foetuses treated with Trypan blue, F6066 or Olive oil.

S bstance	Dosed day	Dose mg/kg	Test No.	Type of malformation	Total number of rats and mice: Foetuses	Total number of rats and mice: Malfor mations
F6066	7–8–9	8	R 1	Small parietal and temporal bones without exencephalon		
	7–8–9	8	R 2	No right pubic bone		
	7–8–9	16	R 2	One ear large and malformed		
	6–7–8	8	M 4	3 sternal bones unsymmetrical		
	7–8	16	M 5	4 sternal bones unsymmetrical	700	5 (0.7)
Trypan blue	6–7–8	100	M 1	Exencephalon, malformed eye		
	6–7–8	100	M 1	Exencephalon		
	7–8–9	*100*	*R 6*	*No tail, anus or lumbar and sacral vertebrae*	*137*	3 (2.2 %)
Control	7–8–9		R 2	Exencephalon } same mother		
	7–8–9		R 2	Exencephalon } same mother		
	1 2		R 5	No right or left pubic bone and 5 sternal bones missing		
	7–8–9		R 6	One ear large and malformed	568	4 (0.7 %)

not show any foetuses. Since pregnancy and non-pregnancy were judged with the naked eye, the result must be regarded as a subjective impression especially when treatment had been started early in pregnancy

In the mice and rats, Trypan blue increased the number of foetal deaths and also produced a slight increase in the frequency of malformations (table 1 R 6 and table 2 M 1) The number and types of gross malformations after Trypan blue F6066 and olive oil are given in table 4 Small differences in the maturity of the skeleton, *e g* number of ribs and breast bones that stained with alizarin is not included because this varied in all the groups.

Discussion

It has been shown that F6066 counteracts the effects of progesterone on the uterus in the rabbit and that the substance accumulates in hormone producing corpora lutea (HANNGREN *et al* 1965 b). In these investigations difficulties were therefore expected since large doses given in early pregnancy would cause abortion. We wanted to use as large a dose of F6066 as possible since if such large doses produced no malformations, small doses would be assumed not to do so either We therefore tried to regulate the doses in such a way as to cause abortion in some of the animals but not in all of them.

During the investigation, we found that the administration of F6066 late in pregnancy also caused a decrease in the number of living foetuses (R 4), if the substance was given uninterruptedly in large doses (16 or 64 mg/kg).

Some animals were treated on 2 consecutive days at varying intervals before implantation others, at the beginning of the organo-poietic period (BROCK & VON KREYBIG 1964) Since treatment of women with F6066 is limited to the time before and (if the diagnosis is not established) to a short time after conception, it was not considered necessary to study its effect, if any in such detail in late as in early pregnancy

Observations made in the present investigation corroborated the finding of HANNGREN *et al* (1965) and of EINER JENSEN (1965) that F6066 has antigestagenic properties. The investigation showed an increased number of dead or resorbed foetuses even in the uteri which also contained living foetuses. This indicates that the effect of F6066 on gestation is not of the "all or none" type.

F6066 did not demonstrably increase the frequency of malformations. In our experiment Trypan blue produced an increase in the number of malformed foetuses from 0 7 / in control animals to 2,2 /. BECK & LLOYD (1964) reported 5–20 / malformed foetuses following correspond-

mg doses of Trypan blue given in a single dose 8½ days after conception. Although they do not state whether the day on which the animals were mated was called day 0 or day 1 they treated the animals during the same period as that used in the present investigation and the frequency of malformations they reported was substantially higher BEAUDOIN & ROBERTS (1965) suggest that the effect of Trypan blue should be sought in serum protein changes in the mother or the foetus, but obtained no experimental evidence to support their assumption on injection of serum protein fractions from animals and pregnant rats treated with Trypan blue. In a discussion between GILLMAN and BECK (1965) the possibility of changes in macromolecules was pointed out, but decreased transport of oxygen in the placenta and changes in the placental enzymes were also mentioned as possible precipitating factors. The cause of the weak effect in our animals is obscure but it may have been due to species differences or to differences in the quality of the Trypan blue used. It can hardly be ascribed to inaccurate or insufficient examination of the foetuses, because the frequency (0 7 %) of severe spontaneous malformations in the control group appeared normal

NISHIHARA (1958) showed that oestrogens (oestradiol and oestrone) increase the foetal mortality and the frequency of cleft palate if the substance is injected into mice between the 11th and 16th day of pregnancy In none of our foetuses was a cleft palate observed in the single experiment (R 4), in which we gave F6066 late in pregnancy but owing to the design of this experiment some malformations might not have been observed since rats often eat malformed offspring.

Triparanol (Mer 29) stunts the growth of young male rats it causes xerophtalmia and reduces the fertility of both males and females, if given to their mothers during pregnancy (WEXLER 1964 ROUX & DUPUIS 1961). A single dose is sufficient to cause this effect, which can be counteracted by simultaneous administration of ACTH The effect is ascribed to the anti-cholesterolic properties of Triparanol. The effect of F6066 on the serum cholesterol is much smaller than that of triparanol (LARSSON, personal communication)

F6066 was not studied for an effect corresponding to that of clomiphene (CHANG 1964 DAVIDSON SCHUNER & WADA 1965) on zygotes and their transport. It can therefore not be decided whether the decreased number of foetuses after treatment with F6066 on days 1–2 and 3–4 is due to the direct effect on the nutrition and transport of the zygotes, to a persistent anti-implantation effect on the uterus, or to a destructive effect on the corpora lutea.

Small but well-developed foetuses were found in 3 mothers treated with F6066 but not in the control group This poor growth should

probably be regarded as an effect of the substance on the decidua and corpora lutea. HANNGREN *et al* (1965b) showed regression of the secretory endometrium after F6066 treatment in immature rabbits and accumulation of the substance or its metabolites in active corpora lutea in mice. LARSSON (personal communication) has observed inhibition of progesterone formation in the rat and human ovaries *in vitro* EINER JENSEN (unpublished) found that F6066 has an antidecidual effect on pseudopregnant rats of the type observed with ethinyl estradiol (WATNICK, GIBSON, VINEGRA & TOLKSDORF 1964) Administration of F6066 for 2–3 days should therefore lead to an insufficient decidua-formation and consequently failure of implantation. An "almost" abortive dose need not mean foetal death, but it can mean decreased nutrition.

The lower weight of the foetuses may also be due to delayed implantation. When rats were ovariectomized on day 2 and then given progesterone daily the blastocystes did not become implanted but implantation did occur when 0.5 µg oestrone was injected. SCHLOUGH & MEYER (1965) showed that anti-oestrogens can delay implantation, but as far as I know this effect has not been described in animals given larger doses of oestrogens or anti-gestagens.

HANNGREN *et al* (1965a & b) showed, that only traces of ^{14}C F6066 or radioactive metabolites pass the placental barrier after intravenous administration to pregnant mice. Therefore a direct effect of the substance on the foetus appears unlikely

The small foetuses, which were sometimes only one third the normal size, cannot be regarded as "congenital runts" (MCLAREN & MICHIE 1960) since they do not occur in association with normal-sized foetuses in the same litter Runts were present in our investigation, as can be seen in figs. 2–4

Summary

Bis(p-acetoxyphenyl)cyclohexylidenemethane (F6066) was studied for its possible antifertility and teratogenic effect, in rats and mice. The substance was given by stomach tube in doses of 2–64 mg/kg dissolved in olive oil. The doses were given on 2 or 3 consecutive days at a varying interval after demonstration of spermatozoa in the vaginal smears in rats or vaginal plugs in mice. In all of the experiments except one, the animals were given the substance on days 1–2, 3–4 5–6, 7–8 or 7–8–9 (rats) and on days 6–7–8 (mice)

When F6066 was given on day 6 or earlier in doses of 32 and 64 mg/kg the mothers hardly ever gave birth to living young. When given in the 3 days period during implantation 16 or 8 mg/kg F6066 disturbed the

pregnancy in some animals, while doses below 8 mg/kg to rats had no effect. In addition to congenital "runts" 1 female rat and 2 female mice treated with F6066 gave birth to 14 living, apparently normal, but severely underweight foetuses (i.e. one third of the weight of control foetuses), but this should be regarded as an effect of the endocrinological properties of the substance. Sub-abortive doses of F6066 produced some increase in the number of foetal deaths. No malformations suggesting that F6066 had a teratogenic effect were demonstrated.

Female rats given F6066 in a daily dose of 16 mg/kg from day 7 of pregnancy to the 5th day of post partum gave birth to normal sized litters and no difference could be demonstrated between the litters of rats of mothers treated in this way and litters from control mothers regarding the size of the sexual organs and the fertility of the offspring. The number of mothers that gave birth to living young was somewhat *smaller after treatment with 16 mg/kg (5/10) and much smaller after 64* mg/kg (1/10) than in corresponding control mothers (9/10).

Trypan blue was used as reference substance, which under the experimental conditions used produced a slight increase in the frequency of malformations in the number of dead foetuses.

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Inhibition by Cardiac Glycosides and Aglycones of the Isolated Guinea Pig Ileum Estimation of Relative Potencies

By

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A method was developed for the quantitative estimation of digitoxin and strophantin-G based on the inhibitory effect on the isolated guinea pig ileum (BIELTVEDT & BRISEID 1966) Previously GODFRAIND & GODFRAIND-DE BECKER had published several papers on the inhibition by cardiac glycosides of the guinea pig ileum (1961 a & b 1962 a & b 1963 a & b) In the present work the method of BIELTVEDT & BRISEID (1966) was used for estimating the activities of a series of cardiac glycosides and aglycones, digitoxin being used as standard substance.

Technique

A. Procedure

The procedure described by BIELTVEDT & BRISEID (1966) was used for estimating the concentrations of a number of cardiac glycosides and aglycones which reduced to 50% submaximal, histamine-produced contractions f the isolated guinea pig ileum. Each result was calculated from the effects obtained simultaneously with digitoxin, which was used as standard substance.

B. Glycoside and aglycone concentrations

Table 1 shows the concentration ranges used of the different cardioactive substances. Each range is based on several experiments.

Stock solutions were made up in 96 % ethanol for most of the substances. The final alcohol concentration in the modified Tyrode solution (BIELTVEDT & BRISEID 1966) never exceeded 0.4 %. Gitoxin, gitaloxin, and verodoxin were dissolved in a mixture of chloroform and methanol (1 + 1 /v). The concentration f chloroform-methanol mixture in the final Tyrode solution never exceeded 0.1 %.

Table 1

Concentration ranges used of different cardiac glycosides and aglycones for the estimation of their inhibiting effects on guinea pig ileum.

Substance	Concentration range ng/ml
Verodoxin.	30–45
Gitaloxin.	35–60
Digitoxin	36–98
Strophanthin-G	100–195
Purpurea glycoside A	125–165
Digoxin	200–300
Gitoxin	200–500
Lanatoside C	450–600
Digitoxigenin	475–675
Digoxigenin	1000–2000
Digitalinum verum	1500–2500
Purpurea glycoside B	1500–2750
Gitoxigenin	2500–4000

C. *Materials*

Purpurea glycoside A (desacetyldigilanid A), digitoxigenin, digitoxin, digoxigenin, digoxin, gitoxigenin, gitoxin, lanatoside C, purpurea glycoside B Sandoz, Ltd., Basle, Switzerland. Digitalinum verum Hoffmann-La Roche & Co. Ltd. Co Basle, Switzerland. Gitaloxin, verodoxin Boehringer & Soehne, GmbH., Mannheim, Germany Strophanthin-G g-Strophanthinum cryst. Ouabain. Merck, A. G Darmstadt, Germany Histamine Histamine di-HCl. Light & Co., Ltd., Colnbrook, England.

Results

Estimations of the ileum-inhibiting effects of 9 cardiac glycosides and 3 aglycones were carried out with digitoxin as standard substance, and the results are given in table 2. This shows the relative potencies of the different substances used. While the activities of verodoxin and gitaloxin were one and a half times that of digitoxin, all the other substances had smaller effect. Purpurea glycoside A and strophanthin-G had more than half the activity of digitoxin, digoxin one-third, and gitoxin and lanatoside C about one-fifth. The remainder of the glycosides and the three aglycones tested had potencies which varied from 1 to 5 / that of digitoxin

Table 2

Inhibition by cardiac glycosides and aglycones of the isolated histamine-stimulated guinea pig ileum.

Estimation of the inhibitory effects of 12 different substances in experiments with digitoxin as standard substance.

Sequence of solutions Experiment I standard and test experiment II test and standard. Incomplete experiments Gitaloxin and verodoxin made the ileum unsuitable for the subsequent application f digitoxin standard solution and the standard values were chosen instead as the average value from all other experiments II and quoted in the table. For details see text and BIELTVEDT & BRISEID (1966)

Cardiac glycosides and aglycones	ng/ml causing 50% inhibition Exp. I		Exp. II		Relative activity Test/Stand. per cent		Average relative activity Test/Stand. per cent	
	Stand.	Test	Test	Stand.	Exp. I	Exp. II	Weight	Molar
Gitaloxin.	72	43	49	65**	167	133	150	159
Verodoxin.	70	32	38	65**	219	171	195	148
Purpurea glycoside A	90	155	140	80	58	57	58	70
Strophanthin-G	83	160	105	54	52	51	52	50
Digoxin	86	230	265	72	37	27	3[illegible]	33
Gitoxin	58	245	450	74	24	16	20	20
Lanatoside C	59	495	550	60	12	11	12	15
Digitoxigenin	67	545	630	61	12	10	11	5
Purpurea glycoside B	66	1550	2425	75	4	3	4	5
Digitalinum verum	71	1860	1950	56	4	3	4	4
Digoxigenin	56	1560	1585	63	4	4	4	4
Gitoxigenin	59	3270	3125	56	2	2	2	1

Comments on the Results

A. *Differences in inhibition by the various cardioactive substances*

The method of assay originally based on digitoxin and strophanthin-G proved less suitable for some of the other substances tested The development and also the persistence of inhibition by digoxin, gitoxin, lanatoside C and purpurea glycoside A were similar to those observed for digitoxin and strophanthin-G with the other glycosides and the aglycones various difficulties were encountered.

The highly active gitaloxin and verodoxin altered the reactivity of the muscle. When the second and stronger glycoside solution had been in contact with the ileum for the prescribed 20 minutes and histamine stimulation was started (BIELTVEDT & BRISEID 1966), the base line often

rose gradually and the contractions became irregular and occasionally more than the normal 11 histamine induced contractions were required to obtain a stable inhibition level.

While the normal development of inhibition was recognized by an increase in inhibition to a stable level in the course of about 11 cycles, digitalinum verum, purpurea glycoside B, and the 3 aglycones very rapidly caused a maximal inhibition, which then more or less rapidly decreased again. With digitalinum verum the final level was obtained after 4–10 contractions, while the inhibition caused by the aglycones and purpurea glycoside B decreased during approximately 20 histamine induced contractions and then reached a stable level In spite of the fact that some substances failed to produce a stable level of inhibition at the usual time, the 11-cycle procedure was nevertheless adhered to as a basis for the calculation of the log-inhibition 50 / values. This was justified by the fact that the substances in question were also found to possess very low activities.

B *Significance of the purity of the cardioactive substances*

The observed activities of the substances tested might have been influenced by contaminating glycosides or aglycones. If low activity substances like the aglycones, digitalinum verum, or purpurea glycoside B were present as impurities in the preparations of the highly active glycosides (verodoxin, gitaloxin, digitoxin) or glycosides of medium activity (purpurea glycoside A, strophanthin-G digoxin, lanatoside C), the activities registered would be somewhat underestimated, though not fundamentally altered. On the other hand, if highly active substances or even substances of medium activity were present as contaminations in the substances with low activity the potencies of the low-activity substances might be greatly overestimated. From a practical point of view it seems of less importance if a low activity as, for example, that of purpurea glycoside B is wrongly attributed to the substance itself instead of to traces of a very active glycoside It is more important, however to be aware that extensive contamination with an active glycoside might wrongly suggest that a particular substance was highly potent. This seems to be the case with the gitoxin preparation used, which by paper chromatography and fluorimetry (BREIEID JENSEN 1954) proved to contain as much as 15 /–20 / of digitoxin. Since the observed activity of gitoxin was 20 / of that of digitoxin, it seems reasonable to conclude that gitoxin itself has only a very low activity This also is in better agreement with the observations that purpurea glycoside B as well as the aglycone gitoxigenin have very low potencies.

Discussion

The procedure used for the quantitative estimation of cardiac glycosides and aglycones might be considered an *in vitro* toxicity test, using the inhibition of the isolated guinea pig ileum, which follows a transitory stimulating phase. The range of potencies established for a number of cardioactive substances, however is not necessarily different from the range which would have resulted from observations of their stimulating effect. BROWN, STAFFORD & WRIGHT (1962) examined a series of derivatives of digitoxigenin and digoxigenin for relative toxicity in the cat, the guinea pig, and on the isolated, embryonic chick heart, and for inotropic activity on the guinea pig isolated heart and the cat isolated papillary muscle. They concluded from their experiments that the order of relative toxicity of the compounds remained essentially the same whether they were tested for toxicity or for positive inotropic activity When considering the results obtained in the *in vitro* assays by BROWN and his colleagues and the comparable data in the present work (digitoxin against digitoxigenin, and lanatoside C and digoxin against digoxigenin), it is evident that the relative potencies obtained with the guinea pig perfused heart (positive inotropic activity) and the embryonic chick heart (toxicity) agreed reasonably well with our results on the isolated guinea pig ileum (toxicity) The sequence of potencies obtained with the cat isolated papillary muscle (positive inotropic activity) however was somewhat different, digoxigenin being more active than lanatoside C.

The order of relative potency of cardioactive glycosides and aglycones as tested both in the present work and in the experiments of DE GRAFF PAFF & LEHMAN (1941) on the embryonic chick heart was also the same.

KLUPP (1966) compared the potencies observed on the guinea pig heart lung preparation and by intravenous infusion into guinea pigs. The substances which we have tested on the isolated guinea pig ileum (digitoxigenin, digitoxin, lanatoside C, strophanthin-G) showed the same potency range as observed in the *in vitro* experiments of KLUPP Moreover the results of CATTELL & GOLD (1941) on the cat isolated papillary muscle also agreed reasonably well with our results.

However ranges of potencies deviating from those obtained by us have been described by other investigators. In experiments on the isolated guinea pig ileum HALUSTEIN MARKWARDT & REPKE (1966) found that gitoxin (chromatographically pure) was almost as effective as digitoxin and somewhat more active than strophanthin-G in increasing the tone of the muscle. Furthermore, in the *in vitro* method described by MURAFE (1965), using frog rectus abdominis muscle, a stronger contracture was always observed with the aglycones than with the corresponding glyco-

sides. The effect, however was of nervous origin, and seemed to be mediated by acetylcholine (SHIGEI, IMAI & MURASE 1963)

Our estimations of cardioactive substances on the isolated guinea pig ileum showed that the gitoxigenin glycosides (B-series) had a very low activity the digoxigenin glycosides (C-series) a considerably higher activity but definitely lower than that of the digitoxigenin glycosides (A series) which were again less active than the gitaloxigenin glycosides (E-series)

The potency of the aglycones digitoxigenin and digoxigenin was markedly increased in the 3-digitoxose glycosides digitoxin and digoxin. An additional glucose molecule, however reduced the activity again to some extent, both purpurea glycoside A and lanatoside C being less active than the corresponding secondary glycosides.

The potency of gitoxigenin was also increased in the 3-digitoxose glycoside gitoxin, which was, however contaminated with digitoxin. The fact that purpurea glycoside B like the corresponding aglycone, showed very low activity pointed to the digitoxin content as mainly responsible for the observed gitoxin effect. The previously mentioned transitory stronger inhibition by the B-series (and aglycones from the other series) might suggest weak affinities as the main reason for their low activities.

Summary

A method developed by BIELTVEDT & BRISEID (1966) for the quantitative estimation of cardiac glycosides by means of their inhibitory effects on the isolated guinea pig ileum was used to estimate the relative potencies of 10 cardiac glycosides and 3 aglycones. Digitoxin was used as standard substance.

The molar order of relative potency was as follows, in decreasing order gitaloxin, verodoxin, digitoxin, purpurea glycoside A, strophanthin-G digoxin, gitoxin, lanatoside C, digitoxigenin, purpurea glycoside B, digitalinum verum, digoxigenin, and gitoxigenin.

When classified according to the aglycone part of the molecules, the decreasing order of relative potency was gitaloxigenin digitoxigenin, digoxigenin and gitoxigenin glycosides.

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Influence of Ca++ Deficiency and Pronethalol on Metabolic and Contractile Effects of Catecholamines and K Ions in Vascular Muscle

By

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In previous investigations (LUNDHOLM & MOHME-LUNDHOLM 1962a & b 1963 & 1965) the effect of catecholamines and K ions on the anaerobic metabolism associated with isotonic and isometric contraction of isolated vascular muscle was studied. It was found that during the shortening phase of the isotonic contraction, the metabolism increased after treatment with catecholamines, histamine and electrical stimulation, and that an increased degree of contraction was subsequently maintained without any metabolic change. K^+ induced an isotonic contraction without stimulating the metabolism. On isometric contraction, however K^+ stimulated the metabolism considerably. This stimulation was in general quantitatively and temporally correlated with the contraction. Under certain conditions it was possible to block selectively the contractile effects of adrenaline and histamine without completely inhibiting their stimulatory actions on the metabolism. It was therefore assumed that in smooth muscle the catecholamines were able to activate the contraction process and the metabolism by two separate mechanisms, the receptors of which were believed to correspond to the classical α and β-receptors. In the following a number of findings which appear to support this hypothesis are presented. We found that isoprenaline, in a concentration that has no contractile effect, stimulates lactate formation in mesenteric arteries. We also studied, under isometric conditions, the effect of Ca^{++} deficiency on the tension-increasing and metabolism-stimulating effects of adrenaline and K. Finally with the aid of pronethalol, we attempted to analyse the mechanism of the stimulatory effects of adrenaline and isoprenaline on metabolism.

Method

The experiments were performed on bovine mesenteric arteries which were treated and mounted as described previously (LUNDHOLM & MOHME LUNDHOLM 1962a). By means of a special holder in which the distance between the points of attachment of the specimen could be measured and varied with micrometer screw all specimens were adjusted to the same initial length at th beginning of the experiments. The isometric tension was recorded on smoked paper. The recording was not absolutely isometric, since at a tension development of 50 g, the muscle shortened by 1 mm.

The lactate contents of the muscle and the suspension solution were determined enzymatically by means of lactate dehydrogenase, as described previously (LUNDHOLM, MOHME LUNDHOLM & VAMOS 1963. LUNDHOLM, MOHME LUNDHOLM & SVEDMYR 1963).

In other respects the different series of experiments were performed as follows. In the first series two specimens were analysed immediately for lactic acid. Four mounted specimens were immersed in 15 ml Tyrode's solution containing 0.5 % glucose and through which N_2 was bubbled adrenaline in concentration of 2.5 10^{-6} (w/v) was added to two of these preparations. Two other mounted specimens were immersed in 15 ml K Tyrode's solution in which all Na ions had been replaced by K ions. After an incubation period of 120 minutes, the specimens and incubation solution were analysed for lactate.

I further experiments the vascular specimens were first kept for 4 days in ice-cold Ca^{++} free Tyrode solution containing 0.1 % glucose and through which 93.5 % O_2 + 6.5 % CO_2 was bubbled. The solution was changed four times during this period. Nine specimens were then mounted and immersed in Ca^{++}-free Tyrode's solution containing 0.5 % glucose and through which N_2, at 37° was bubbled. After 60 minutes two specimens were analysed for their initial content f lactic acid, and the suspending solutions of the remaining specimens were changed. New solutions were added, with such compositions that different combinations of adrenaline, Ca^{++} and K were obtained. All the solutions contained 0.5 % glucose and N_2 was bubbled through them. A control specimen was suspended in Ca^{++}-free Tyrode's solution containing 0.5 % glucose. The remaining specimens were treated as follows: one was suspended in Ca^{++}-free Tyrode's solution + 2.5 10^{-6} (w/v) adrenaline one in high K (145 mEq K /l) Tyrode's solution which was free from Ca^{++} one in normal Tyrode' solution (with 2.5 mEq Ca^{++}/l) one in normal Tyrode's solution + adrenaline one in high K Tyrode's solution containing Ca^{++} and finally one in high K Tyrode's solution containing Ca^{++} + adrenaline. After an incubation period f 120 minutes, the specimens and suspending solutions were analysed for lactate.

In the experiments with pronethalol and adrenaline, three of six specimens were pretreated for 60 minutes with pronethalol in concentration of 5 10^{-5} (w/v) in Tyrode's solution with 0.5 % glucose. The initial lactat content was determined in one untreated and one pronethalol-treated specimen, and the suspending solutions of the remaining specimens were changed. Adrenaline in a concentration of 2.5 10^{-6} (w/v) was added to one of the specimens, pronethalol 5 10^{-5} (w/v) to another and drenaline + pronethalol i these concentrations to third specimen. One specimen was used as a control. N_2 was bubbled through the solutions.

The experiments with DL-isoprenaline and pronethalol were carried out i the same way a those with drenaline and pronethalol. The isoprenaline concentration was 1 10^{-6} (w/v) and the pronethalol concentration 1 10^{-5} (w/v).

Results

The effect of Ca^{++} deficiency on the contractile and metabolic effects of adrenaline and K-ions

Several investigators have shown that with lack of Ca^{++} ions, the contractile response of smooth muscle to different stimuli is inhibited (EDMAN & SCHILD 1961 BOHR & GOULET 1961) In K^{+}-depolarized smooth muscle the uptake of Ca^{++} is also reported to be increased (BRIGGS 1962). The Ca^{++} ion has been considered to be the connecting factor between the excitation process in the muscle membrane and the contraction process It therefore seemed of interest to study the influence of Ca^{++} deficiency on the contractile and metabolic effects of adrenaline and K^{+}

It was difficult, in the mesenteric artery to produce such a high degree of Ca^{++} deficiency so as to eliminate completely the contractile effect of adrenaline and K^{+} This was probably due to the fact that large vessels contain considerable quantities of Ca^{++} this content increasing with increasing age (LAUSING 1959) It was not until the specimen had been suspended in Ca^{++} free Tyrode s solution for 4 days that the contractile effect of adrenaline became appreciably weakened

The results are shown in table I and fig. 1 In Ca^{++} free Tyrode s solution the average effect of adrenaline on the tension during a period of 120 minutes was reduced to $\frac{1}{4}$ of that in normal Tyrode s solution, while the effect on lactate production was only reduced to $\frac{3}{4}$ of this value.

In Ca^{++}-free solution the tension-increasing effect of the K^{+} ion was reduced to approximately $\frac{1}{4}$ of that in normal Tyrode s solution. The tension-increasing effects of K^{+} and adrenaline in Ca^{++} free solution were approximately equal, but the increase in metabolism after treatment with adrenaline was considerably greater The comparatively large increase of the metabolism produced by adrenaline in relation to its tension increasing effect, appeared to be particularly marked when compared with the tension- and metabolism-increasing effects of the combined K and Ca-ions. In these latter experiments the tension increase was among the highest observed in this series, while the increase in metabolism was among the lowest (fig. 1) The stimulating effect of adrenaline on the metabolism was very pronounced throughout, as compared with its tension-increasing effect.

Ca^{+} ions stimulated the tension and metabolism in specimens which had been suspended in Ca free solution. In ordinary Tyrode s solution (containing Ca^{++}), a further addition of Ca^{++} had no effect. In combination with K-ions, Ca-ions had no definite stimulating effect on the metab-

Table 1

Influence f adrenaline (2.5 10⁻⁶), K (145 mEq/l) and Ca^{++} (2.5 mEq/l) on the *lactate production* (LAP) in mg/100 g tissue/120 min. and mean increase f tension in g wt/120 min. in tests on isolated bovine mesenteric artery suspended in normal Tyrode s solution and Ca^{++}-free Tyrode s solution with 0.5 per cent glucose through which N_2 was bubbled. Mean of 5 tests. P = probability that the effect was due to chance.

Preparation suspended I	Basal LAP	Increase in LAP after addition of adr	Ca^{++}	K	adr + Ca^{++}	adr + K	adr + Ca^{++} + K	Ca^{++} + K
Tyrode (normal)								
LAP	285 ± 20	198 ± 41 P < 0.001		95 ± 26 P < 0.05				
Tensio	0	22		27				
Ca^{++}-free Tyrode								
LAP	214 ± 33	150 ± 23 P < 0.01	111 ± 20 P < 0.01	66 ± 34	297 ± 21 P < 0.01	141 ± 53 P < 0.05	135 ± 88	42 ± 18 P < 0.05
Tension	0	5.0	5.0	6.0	25	18.5	26.5	22
Ca -free K Tyrode								
LAP	279 ± 22	75 ± 23 P < 0.02	−23 ± 21	–	70 ± 32 P < 0.05	–	–	–
Tension	6.0	18.5	5.0		25.0			

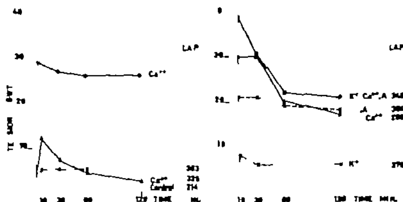


Fig. 1 The effect of 2.5 10^{-6} (w/v) adrenaline (A), 2.5 mEq/l Ca++ 145 mEq/l K separately or in combination, on the tension and lactate production in mg/100 g muscle/100 min. (LAP) of bovine mesenteric artery under anaerobic conditions in Ca++-free Tyrode's solution with 0.5 % glucose t 37° The specimens had initially been suspended in Ca++ free oxygenated Tyrode solution at 4 for 100 hours. Control — test in Ca++-free Tyrode's solution. Each point represents the mean of 5 tests.

olism, while on the other hand, the tension-increasing effect was very marked

Adrenaline combined with K however stimulated both the metabolism and tension. The presence or absence of Ca++ had no effect on the increase in metabolism after adrenaline and K+ in combination while the tension increase, on the other hand, was greater in the presence of Ca++ In ordinary Tyrode s solution, however the effects of adrenaline and Ca++ on both the metabolism and tension were summated. It is probable that Ca++ increases the metabolism and tension in vascular tissue that has been suspended in Ca++-free solution, but that the stimulating effect of the Ca++ on the metabolism is inhibited in high-K solution

The influence of pronethalol on the stimulating effect of adrenaline on contraction and metabolism

Pronethalol (alderlin ® ICI) is an adrenergic β-receptor blocking agent, which has been found to inhibit the relaxant effect of adrenaline and isoprenaline on smooth muscle as well as the stimulating effect of these amines on the heart (BLACK & STEPHENSON 1962). It has also been found, however to have both β-stimulating and α-blocking effects (LISH, WEIKEL & DUNGAN 1965). Pronethalol inhibited the phosphorylase-activating effect of adrenaline and isoprenaline in experiments on the rat diaphragm (MOHME-LUNDHOLM & SVEDMYR 1964).

Table 2

The influence of adrenaline, isoprenaline and pronethalol on lactate production (LAP in mg/100 g/120 min.) and tension in part of bovine mesenteric artery Mean of 5 tests. P = probability that the effect was due to chance.

Experimental conditions	Control	Change of LAP from control after			Change of LAP from pronethalol tests	Maximal increase of tension after	
		adrenaline	isoprenaline	pronethalol	pronethalol + adrenaline	adrenaline	pronethalol + adrenaline
Adrenaline 2.5 10^{-4} Pronethalol 5 10^{-} in N_2 (n = 5)	227 ± 30	331 ± 26 $P < 0.001$	–	69 ± 27 $P < 0.05$	11 ± 22	23	0
Adrenaline 2.5 10^{-4} Pronethalol 5 10^{-4} in O_2 (n = 5)	32 ± 7.2	40 ± 7.6 $P < 0.01$	–	−20 ± 12.3	21 ± 10.6	32	11
					pronethalol + isoprenaline	isoprenaline	pronethalol + isoprenaline
Isoprenaline 1 10^{-} Pronethalol 1 10^{-3} in N_2	259 ± 25		159 ± 36 $P < 0.01$	−142 ± 31 $P < 0.01$	79 ± 30 $P < 0.05$	0	0

Difference in adrenaline effect without and with pronethalol in N_2 320 ± 34 $P < 0.001$
Difference in isoprenaline effect without and with pronethalol in N_2 80 ± 46.

In a concentration of 5 10 5 (w/v) pronethalol *per se* stimulated lactate production of the vascular specimen In a concentration of 1 10 3 (w/v), on the other hand, it inhibited this production, after an initial stimulation.

Under anaerobic conditions the effect of adrenaline in a concentration of 2.5 10^{-6} (w/v) on the metabolism and contraction was completely eliminated by pronethalol in a concentration of 5 10^{-6} (w/v) (table 2). Under aerobic conditions pronethalol in the same concentration reduced the contractile effect, alone, of adrenaline from a maximum of 32 to 11 gwt. It seems probable that the α-blocking and β-stimulating effects of pronethalol are of importance in these experiments.

The effect of isoprenaline on lactate production of the vascular specimen under anaerobic conditions

DL-isoprenaline in concentrations of 5 10^{-6} to 1 10^{-4} (w/v) stimulated the anaerobic lactate production of the vascular specimen without influencing its tension (table 2) In a concentration of 5 10^{-4} (w/v) it was found that it not only stimulated the metabolism but also had a contractile effect. Pronethalol, which in itself inhibited the formation of lactate, also reduced the effect of isoprenaline on lactate formation in a concentration of 1 10^{-4} (w/v) Mesenteric artery which had been contracted by K^{+} was relaxed to some extent by isoprenaline

Discussion

It would seem that the stimulating effect of catecholamines on the lactate production of vascular muscle can be divided into two components, a) direct or primary stimulation of the metabolism and b) secondary stimulation resulting from the contraction process. In support of this assumption is the fact that isoprenaline increased the lactate production by about 160 mg per 100 g per 120 min. without simultaneously contracting the vascular muscle. The stimulating effect of adrenaline, which in normal vascular tissue was found to correspond to about 200 mg, was reduced to about 150 mg, when the contractile effect was inhibited in Ca^{++}-free solution.

These experiments indicate that the primary stimulation of the metabolism of vascular muscle by catecholamines corresponds to about 150 mg, while the effect resulting from the contraction process may be estimated as 50–100 mg depending on the size of the tension increase. This assumption is further supported by the finding that in normal Tyrode's solution, the increase in lactate production caused by adrenaline was higher than that induced by K in spite of the fact that the initial tension increase after treatment with K was greater

Pronethalol almost completely inhibited the stimulating effect of adrenaline on the metabolism. However pronethalol also blocked the tension increasing effect of adrenaline and in itself stimulated the formation of lactate. Thus in these experiments, pronethalol did not act as a sufficiently specific adrenergic β-blocking agent, so that definite conclusion cannot be drawn as to whether or not adrenaline exerted its primary effect on the metabolism via stimulation of adrenergic β-receptors however this does seem probable.

In vascular specimens which had been suspended in Ca^{++}-free solution, Ca^{++} appeared to stimulate the metabolism by means of a mechanism which was not directly correlated with the contraction process. The effect of Ca^{++} on the metabolism was blocked by K^{+} In specimens which had been suspended in Ca^{++}-free Tyrode s solution, Ca^{++} ions increased the lactate production by about 110 mg per 100 g per 120 min., and at the same time the muscular tension increased somewhat. In K^{+} Tyrode s solution the development of tension after Ca^{++} was greater but the increase in metabolism was significantly less (69 ± 17 mg/100 g/120 min., $P < 0.01$) A similar result was also obtained in the presence of adrenaline. The combined effect of Ca^{++} ions and adrenaline on the metabolism (297 mg) was approximately equal to the sum of their separate effects (Ca^{+} = 111 mg adrenaline = 149 mg) In K Tyrode s solution, where the increase in tension after treatment with Ca^{++} and adrenaline was equal to that in Na^{+} Tyrode s solution, the combined effect on the metabolism was no greater (135 mg) than the effect of adrenaline alone in K^{+} Tyrode s solution (140 mg) The mechanism by which Ca^{++} stimulates the metabolism in vascular muscle is unknown The Ca stimulating ATP ase in the sarcoplasmic reticulum, demonstrated by HASSELBACH & MACKINOSE (1961) may conceivably be of importance for this effect.

It is probable that adrenaline, and possibly also Ca^{++} stimulated the metabolism in the vascular muscle both via the contraction process and by more direct pathways. The energy consumption associated with the contraction of vascular muscle may therefore be easily overestimated if this probability is not taken into account. By determining the changes in the contents of high energy phosphate compounds during the first minute of an isometric contraction we estimated that the development of tension in vascular muscle required an approximately equal amount of energy to that in skeletal muscle (BEVIZ *et al* 1965) The energy required to maintain a certain tension level cannot be determined, however solely from the consumption of high energy phosphate compounds, but with anaerobic conditions the lactate production must also be included in the calculations. The possibility that this may be stimulated

via other mechanisms than the contraction process must thus be considered. It would be of value to use, if possible, an agent which would stimulate the contraction process selectively without influencing the metabolic processes by other means. In vascular muscle K^+ seems to be the agents which comes nearest to fulfilling this condition. In these experiments K^+ had the weakest stimulating effect on the metabolism in relation to its contractile action. Previous experiments with K^+ have also given similar results (LUNDHOLM & MOHME-LUNDHOLM 1962 & 1965). On the other hand, it can be seen in fig. 1 that only in the presence of adrenaline did the vascular muscle maintain a maximal tension level. In other cases the tension decreased progressively. It is therefore possible that the direct stimulating effect of adrenaline on the metabolism was *of importance for the maintenance of a maximal contraction.*

Summary

In experiments on isolated bovine mesenteric arteries, the influence of adrenaline and K-ions on the lactate production associated with isometric contraction was determined under anaerobic conditions. In Ca^{++} free solution the contractile effect of the adrenaline was considerably weakened, while the metabolism-stimulating effect was reduced to a proportionately lesser degree. Ca^{++} increased both the tension and the metabolism in vascular specimens which had been suspended in Ca^{++}-free solution. When combined with K^+, however, Ca^{++} only increased the tension, the stimulating effect of Ca^{++} on the metabolism being blocked by the K^+. Adrenaline in combination with K stimulated both the tension and metabolism. Isoprenaline in concentrations which had no contractile effect stimulated the metabolism in ordinary Tyrode's solution. The metabolism-stimulating effect of adrenaline, and to a certain extent that of isoprenaline, was inhibited by pronethalol, which also blocked the contractile effect of adrenaline under anaerobic conditions. The results obtained are discussed and considered in the light of a hypothesis presented previously that catecholamines stimulate the metabolism in *smooth muscle both via a direct mechanism and via the contraction* process.

Acknowledgements

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Noradrenaline in Human Blood Plasma and Urine During Exercise in Patients Receiving Large Doses of Chlorpromazine

By

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Chlorpromazine is widely used in psychiatry and the range of doses used varies considerably In mental disease with severe symptoms the dose may be kept as high as several grams per day for months or years. Side-effects have been reported – circulatory effects, neurological disturbances, pigment deposits in the skin and eye and, occasionally sudden death (e.g. GREINER & BERRY 1964 GREINER & NICOLSON 1964 DENCKER & ENOKSSON 1966)

Most reports on the effect of chlorpromazine are based on series of patients given a single dose or short term treatment. This is also true for several other drugs which are also used clinically for long periods and in large doses. However there is evidence that the effects of chlorpromazine may change with time The circulatory and physiological effects of chlor promazine, for example, have thus been studied before and up to 90 days during the administration (SLETTEN, LANO BROWN, BALLOU & GERSHON 1965 see also for review)

The mode of action of chlorpromazine is complex, but many demon strable actions of the drug seem to be secondary to effects on the catecholamine receptors in the peripheral and central nervous system.

During physical exercise the activity of the sympathetic nervous system is increased. This raises the level of noradrenaline (NA) in the blood (e g. VENDSALU 1960) and in the urine (v EULER & HELLNER 1952) since the transmitter NA, released at the nerve endings, is carried off by the blood stream ("overflow") The NA levels in the plasma and urine may under some conditions (A. CARLSSON, FOLKOW & HÄGGENDAL 1964), reflect the activity of the sympathetic nervous system, "the tone in the adrenergic neurons" It should be easier to demonstrate the effect of

Table 1

Plasma levels of noradrenaline (NA) at rest and during muscular work.

Case	Age	Duration of disease years	Chlorpromazine dose mg	Chlorpromazine duration months	Other drugs: drug	Other drugs: dose	Other drugs: duration	NA µg/l plasma: at rest	NA µg/l plasma, load kpm/min.: 300	600	(750–)900
No chlorpromazine, untrained											
A	37	20						0.7	1 3	2.6	2.9
B	19	3						0 8	1 4	2.3	2.7 (750)
C	31	6			amylobarbitone	100 mg	short period	0.5	1.3	2.6	
D	4	20			phenytoin	100 mg × 3	>2 months	0.7	1 3	2.1	
					phenobarbitone	100 mg × 3	>2 months				
E	28	6						0.3	0.6	–	4 0
F	24	3						0.8	1.0	1 1	1 4
G	34	15						0.3	0.5	–	2.3
H	56	16						0.8	0.8	0.6	1 4
I	55	4						0.6	1.8	2.7	4 0
							Mean ± S.E.M	0.61 ± 0.068	1 11 ± 0.140	2.00 ± 0.312	2.67 ± 0 407
No chlorpromazine, trained											
A	37	20			Agryban ®	500 mg	1 month 1 night, if needed	0.6	1.2	1.8	2.0
B	19	3						0.6	1.2	0.9	1.3 (750)
C	31	6			amylobarbitone	100 mg	short period	0.4	0.6	0.9	2.9
D	42	20			phenytoin	100 mg × 3	>2 months	1 0	1 1		
					phenobarbitone	100 mg × 3	>2 months				
							Mean ± S.E.M	0.65 ± 0.126	1.03 ± 0.144	1.20 ± 0.300	2.07 ± 0.464

With chlorpromazine, untrained											
E	23	6	2000	1.5	amphetamine	20 mg × 2	1 months 10 days	1.1	1.1	5.5	7.0
J	43	9	3600	>12				2.0	5.4	15.0	
K	26	6	2300	4				0.9	4.1	4.5	
L	23	3	1800	2				0.8	3.9	3.9	
M	27	5	2400	>12	benztropine	2 mg × 1	>2 months	1.5	4.7	2.7	
					Eff til ®	20 mg × 3	>2 months				
N	23	2	1500	>12	benztropine	2 mg × 1	>2 months	0.9	1.5		
					Effortil ®	5 mg × 3	1 month				
O	50	8	2100	>12	benztropine	2 mg × 1	>2 months	1.0	3.2		
					amphetamine	5 mg × 2	>2 months				
							Mean ± S.E.M.	1.17 ± 0.163	3.70 ± 0.654	6.72 ± 2.14	7.0
With chlorpromazine, trained											
C	31	6	900	4	amphetamine	5 mg × 2	9 days	0.9	1.5	3.7	7.9
					Effortil ®	10 mg × 3	>2 months				
G	34	15	1200	1.5				0.8	2.2	2.9	4.7
J	43	9	3600	>12				1.0	2.0	5.4	
K	6	6	2500	8	amphetamine	10 mg × 2	20 days	1.7	2.3	–	3.3 (750)
L	23	3	1800	5	Effortil ®	5 mg × 3	5 days	1.3	5.4	7.1	9.0
M	27	5	2400	>12	benztropine	2 mg × 1	>2 months	0.1	1.2	1.3	
					Effortil ®	20 mg × 3	>2 months				
P	31	11	3000	8	benztropine	2 mg × 1	>2 months	0.5	0.8	1.3	2.3
					Aprobet ®	25 mg × 3	>2 months				
					amphetamine	10 mg × 2	>2 months				
					diethylpropion	25 mg × 3	1 month				
							Mean ± S.E.M.	0.90 ± 0.196	2.20 ± 0.572	3.62 ± 0.941	5.44 ± 1.30

drugs on adrenergic mechanisms, when adrenergic tone is increased than when it is at rest.

In the present study the NA levels in plasma and in some cases also in urine were estimated before and during physical exercise on a bicycle ergometer in patients who for months had received large doses of chlorpromazine because of severe mental symptoms. Preliminary results have been published previously (C. CARLSSON, DENCKER, GRIMBY & HÄGGENDAL 1965 & 1966).

Material and Methods

The material consisted of 16 male inpatients (aged 19–56 years) of a mental hospital. They all had some chronic mental disease, usually schizophrenia. The patients in one group had received about 1–3 g of chlorpromazine a day for at least about two months, and usually more than six months. The other patients had not received any chlorpromazine. It was not possible, however, to keep all the patients completely free from other drugs (table 1 and 2). Most of the patients took part in a course of physical training for rehabilitation purposes. The patients were studied before and after some months of training on a bicycle ergometer. Most of them had previously been more or less physically inactive for years.

The biochemical and physiological methods used have been described elsewhere (HÄGGENDAL 1963, HOLMGREN *et al.* 1957, ÅSTRAND 1960, ÅSTRAND & SALTIN 1961).

The subjects were allowed to rest for 30 minutes before the physical exercise, which consisted of cycling in the sitting position. The load on the bicycle ergometer was increased stepwise to 300, 600 and if possible, 900 kpm/min. The patient was tested for 10–12 minutes with each load. Between these periods the patient was allowed to rest for 20 minutes. At rest and at the end of every test period, arterial blood samples (18 ml) were taken for catecholamine assay. Urine was occasionally collected before and after exercise.

Results

The effect of large doses of chlorpromazine, particularly on physical performance, was marked. The patients treated with chlorpromazine had a lower arterial blood pressure during exercise than the untreated subjects. In most cases increases in the load on the ergometer were followed by decreases in the mean arterial blood pressure and usually a small stroke volume associated with a relatively low cardiac output. As a rule, physical training for 2–4 months decreased the heart rate and the blood lactic acid concentration at submaximal work loads [1]).

The plasma NA levels are given in table 1 and fig. 1. In the patients not treated with chlorpromazine the plasma NA increased during exercise. The increase was about the same as that found by VENDSALU (1960) in

[1]) Data about the circulatory effect of chlorpromazine and of physical training will be published in detail later.

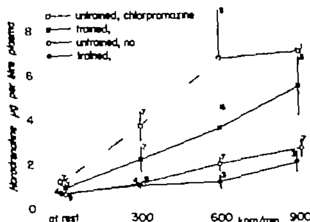


Fig. 1 Plasma levels of noradrenaline (mean, S.E.M. and number of observations) at rest and during muscular work in patients with and without chlorpromazine.

healthy subjects. The resting levels were, however somewhat higher than those given by VENDSALU (1960) and HÄGGENDAL (1963) This may be explained by the mental state of the patients. In the untreated schizophrenics the NA during exercise tended to be slightly lower after the patient had undergone a course of physical training.

In the patients on high doses of chlorpromazine the mean resting NA level was higher than in the untreated patients, and it rose faster during exercise In some cases very high plasma values were noted, e.g. 7µg NA/l plasma or more in four cases and 15 µg NA/l in one case. The tendency of the NA to be lower after physical training was also found in the patients treated with chlorpromazine.

Blood samples collected during rest or exercise contained no demonstrable amounts of adrenaline.

At rest only small amounts of adrenaline were demonstrated in the urine and the increase of catecholamines after exercise was mainly due to NA (table 2). There were several difficulties in collecting the urine some patients could not urinate at the selected time and the volumes collected from others were sometimes not large enough for satisfactory analysis. For more detailed studies catheterization is probably necessary

However at rest as well as after exercise the NA values in the urine were higher in the patients treated with chlorpromazine than in those not treated whether the patients had or had not received any other drugs. All these patients received ascorbic acid.

One neurotic inpatient, male, age 46, physically untrained and given no chlorpromazine, received for 6 weeks some of the additional drugs given to some patients on chlorpromazine treatment, i.e. n-ethyl-nor

Table 2

Urinary excretion of noradrenaline (NA) (and adrenaline (A)) at rest and during muscular work.

	Other drugs			Time t rest	Urine volume	Time at work	Urine volume	Excretion of NA (+A)	
								At rest	During exercise
	drug	dose	duration	min.	ml	min.	ml	µg/min.	
	(ascorbic acid in ll the cases)	(300 mg)	(16 days)						
Without chlorpromazine									
Case 1				20	23.5	12	112	43	182
Case 2				20	50	18	100	38	62
Case 3				40	12	19	225	3	124
With chlorpromazine									
Case 4	benztropine	2 mg	> 6 months	20	150	18	130	134	234
	Aprobit ®	25 mg × 3	> 6 months						
	methylprylon	200 mg	> 6 months						
Case 5				20	100	18	150	132	276
Case 6	Effortil ®	5 mg × 3	> 6 months	20	18	12	100	45	272

One case in every group was excluded, since the urine volume was very low i.e. not above 10 ml.
) The figures are calculated NA

phenylephrine- hydrochloride (effontil ®) 5 × 3 mg, benztropine 2 mg, and amylobarbitone 100 mg for short periods. The plasma levels of NA at rest were 0.5 µg/l and during muscular work with a load of 300 600, and 900 kpm/min. 0.5 0.6, and 1 7 µg/l, respectively The increase in the NA values was thus not as high as in most cases on chlorpromazine treatment, but within the range of patients not treated with chlorpromazine.

Discussion

The results in the case described above and the results of the urinary estimations, as well as the fact that the high NA values were obtained in patients receiving chlorpromazine, often as the only drug, makes it unlikely that the increase in the NA values was due to these other drugs. It rather seems that some of these other drugs might sometimes have decreased the NA values (cases M, N and P)

The coexistence of a decreased effect of the sympathetic nervous system on the circulation and high plasma NA levels, suggesting high sympathetic acitivity can be explained by the fact that chlorpromazine blocks the adrenergic receptors.

The increased concentration of NA in patients treated with chlor promazine may be explained by some different mechanisms

1) Chlorpromazine has a blocking effect on one of the most important inactivating mechanisms at the nerve terminal-receptor level i.e. it blocks the reabsorption of the liberated transmitter into the nerve terminal (HERTTING, AXELROD & WHITBY 1961 AXELROD HERTTING & POTTER 1962 DENGLER, SPIEGEL & TITUS 1961 GEY & PLETSCHER 1961 MUSCHOLL 1961 MALMFORS 1965).

2) It cannot, however be excluded that other inactivating mechanisms were affected by chlorpromazine in these large doses. The circulating NA is mainly metabolized by 3-0-methylation in the liver (see AXELROD 1959). It is possible that chlorpromazine makes it more difficult for the circulating NA to penetrate into the liver cells. It may be recalled that chlorpromazine has an effect on the liver – one of its side-effects being liver damage The high NA levels in plasma and urine might be explained in this way but an effect on the liver is probably only a contributory cause. Studies on the total amount of NA metabolites in the urine during exercise would therefore be of interest.

3) Since the normal effect of the sympathetic activity was reduced by the receptor blockade, the activity of the sympathetic nervous system may be increased by a compensatory mechanism resulting in an increased level of plasma NA If this is the most important mechanism, other drugs affect

ing the effector cells, e.g., receptor blocking agents, should increase the plasma NA level.

4) There may be a direct stimulating effect of chlorpromazine on NA synthesis.

It is possible that the increase in the NA in plasma and urine requires long-term treatment with large doses of the drug, but investigations on the effect of different doses of chlorpromazine, given for a short time, are desirable. Preliminary results on the effect of single doses of chlorpromazine given intravenously have so far shown that the NA concentrations during exercise are of the same order as those found without the drug.

Both the plasma NA level and the urinary excretion of NA were higher in patients receiving chlorpromazine Whether mechanism 1 or 3 is the most important one involved, the increased loss of NA must have been compensated for by an increased synthesis of NA since the stores of NA in the nervous tissue appear to be limited Treatment with chlorpromazine appears to be accompanied by an increase in the catecholamine synthesis in the central nervous system (A CARLSSON & LINDQVIST 1963 ANDÉN, ROOS & WERDINIUS 1964)

An increase in the NA synthesis implies that the synthesis of its precursors must also be increased since the tissue does not appear to contain stores of e.g. DOPA or tyrosine. It is tempting to assume that the possibly increased synthesis of precursors of catecholamines might also result in an increased formation of melanine or melanine-like compounds, since both substances have common precursors. If so, the pigmentation observed after treatment with high doses of phenothiazine drugs may be related to the catecholamine metabolism There may however also be a direct stimulating effect by phenothiazine drugs on the formation of DOPA from tyrosine.

In some cases the NA level during exercise was very high, i.e. above 7 µg NA/l plasma. Such high values are seldom obtained with modern assay techniques except in cases of phaeochromocytoma and then often in connection with severe circulatory disorders. During chlorpromazine therapy the receptor blocking effect of the drug apparently protects the subject against such side-effects However the balance between receptor blockade and high NA levels may be disturbed Furthermore, chlorpromazine does not block all receptors to the same extent Chlorpromazine is chiefly an α-receptor blocking agent but all α receptors may not be blocked to the same extent. Some of the metabolic effects of chlorpromazine may be due to the large amounts of NA present. The cases of sudden death reported (GREINER & NICOLSON 1964 HOLLISTER & KOSEK 1965) may be due to very high catecholamine plasma levels, obtained e.g. during physical activity

Summary

In patients treated with large doses of chlorpromazine, the blood nor adrenaline levels during exercise were high The concentration of nor adrenaline in the urine before and during exercise was also higher in chlorpromazine treated than in untreated patients.

Possible mechanisms are discussed

Block of the reabsorption of liberated transmitter into the nerve terminal.

Effect on the catecholamine metabolism in the liver

Compensatory increased activity in the peripheral adrenergic nervous system due to the α-receptor blocking effect of chlorpromazine

Direct stimulating effect of chlorpromazine on the synthesis of nor adrenaline.

Some side-effects of chlorpromazine treatment are discussed with regard to the effect of chlorpromazine on the catecholamine metabolism

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Pharmacology of Reserpine Analogues VI. Reserpine Analogues with Differential Effect on Brain Monoamines

By

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The central depressant effect of reserpine is accompanied by a decrease in the brain monoamines 5-hydroxytryptamine (5-HT), noradrenaline (NA) and dopamine (DA) The dose-response curves of monoamine levels in short as well as in long-term experiments are rather similar (Carlsson 1959 1960 & 1964 Häggendal & Lindqvist 1963 Markiewicz 1963) However the behavioural changes can not be closely correlated to the extent of monoamine decrease (Häggendal & Lindqvist 1963 & 1964 Markiewicz 1963)

Reserpine like compounds have been described which affect the 5-HT less than the catecholamines and it has been suggested that their sedative action is related to 5-HT (Brodie, Finger, Orlans, Quinn & Sulser 1960). However there is no definite evidence of correlation between the tranquillizing effect of reserpine and the degree of depletion of particular monoamines. Four reserpine analogues have been described, in which a dissociation of sedative and hypotensive activity in monkeys has been determined The ratio of minimal hypotensive to minimal sedative doses in monkeys is much less than 1 (fig. 1) For reserpine this ratio is approximately 1 (Trčka, Dlabač & Vaněček 1963 & 1966). In the present study the effect of some of these substances on monoamine levels in the brain and heart have been investegated in mice and correlated to the spontaneous motility and ptosis (for preliminary report, see Trčka & Carlsson 1965).

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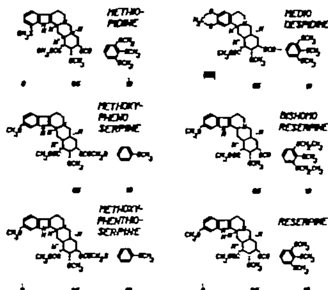


Fig. 1 Chemical structure of tested substances and the ratio between their hypotensive and sedative activities in monkeys. Horizontal bars the ratio between the minimal hypotensive and sedative dose in monkeys in comparison with reserpine. Substances with ratio lower than 1 have relatively higher hypotensive than sedative effects (TRČKA, DLABAČ & VANĚČEK 1966)

Material and Methods

The following derivatives were chosen for this study (fig. 1) *Methiopidine* 1 -methylmercaptodeserpidine (ERNEST 1964) *methoxyphenoserpine* ( )-methyl-0-(p-methoxyphenoxyacetyl)-reserpate (ERNEST & PROTIVA 1963) *methoxyphenthioserpine* ( )-methyl-0-(p-methoxyphenylmercaptoacetyl)reserpate (JIRKOVSKÝ ERNEST & PROTIVA 1965) *medio-despidine* methylene-dioxy-despyrrolodeserpidine (JIRKOVSKÝ & PROTIVA 1963) The effect of these substances was compared with that of *bishomoreserpine*, ( )-methyl-0-(3,5-diethoxy-4-methoxybenzoyl)-reserpate (ERNEST & PROTIVA 1963) an analogue with a reserpine-like sedative effect. Reserpine was used as reference standard.

All substances were dissolved in mixture of dimethylacetamide, propyleneglycol and 5% solution with addition of citric acid. The solution was administered intraperitoneally in volume of 0.5 ml/20 g. A corresponding amount of the solvent was given to the control animals.

Experiments were carried out on female albino mice weighing 20–22 g.

The monoamine content in mouse tissues was determined in pooled samples of six animals. NA in the brain and heart was determined according to BERTLER, CARLSSON & ROSENGREN (1958) and DA in the brain using the method of CARLSSON & WALDECK (1958). The method of BERTLER (1961) was used for the determination of 5-HT levels in tissues.

The effect of the tested substances on spontaneous motility was studied in groups of six mice. The animals in each individual group were placed in another cage. After one minute which was the time allowed for adaptation to the new environment, the number of actually moving mice was noted repeatedly in ten second intervals for two minutes.

The highest possible total number of movements was thus 72 in one session. In repeated experiments, the score of control groups persisted within the range of 68–72. The motility of an experimental group was expressed in terms of per cent of the corresponding control group score.

The width of the eyelid slot was arbitrarily evaluated in four degrees.

All drugs were administered in equi-effective hypotensive doses estimated from previous experiments in unanesthetized normotensive monkeys (TRČKA, DLABAČ & VANĚČEK 1963 & 1966) as follows: methiopidine 1.5 mg/kg, methoxyphenoserpine 7.5 mg/kg, methoxyphenthioserpine 7.5 mg/kg, mediodespidine 5 mg/kg, bishomoreserpine and reserpine 1.25 mg/kg. Monoamine levels and other functions were determined 3 h after administration.

Results

As shown in fig. 2 all substances brought about a marked decrease of heart NA 3 h after administration. After reserpine or bishomoreserpine treatment (both 1.25 mg/kg) almost complete ptosis and disappearance of spontaneous motility were found. The brain 5-HT was decreased to 30–35 % of the control level. The lowering of the catecholamine content in the brain was much more marked. A less-marked decrease in brain monoamines was observed in mice given drugs that did not influence spontaneous motility. In particular the 5-HT level was only slightly affected. The difference between the monoamines was very marked in the mediodespidine group. NA as well as DA were reduced by more than 50 % while 5-HT was only slightly affected. With the exception of metoxyphentioserpine, no effect on the eye aperture width was observed with this group of substances.

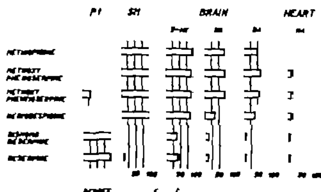


Fig. 2. The effect of tested substances in equi-effective hypotensive doses (in monkeys see text) on the width of the eyelid slot (Pt), spontaneous motility (SM) and monoamine content in mouse organs.

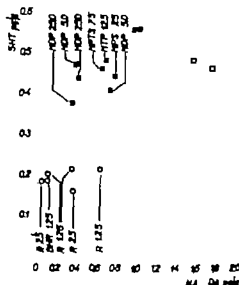


Fig. 3 The 5-HT level plotted against the catecholamine content (NA + DA) in mouse brain 3 hours after administration of tested substances.
White squares control values.
Black squares monoamine levels after administration of analogues lacking any sedative effect.
White circles monoamine levels after administration of reserpine and bishomoreserpine. Marked sedation in all animals.

The disproportion in the decrease of 5-HT and catecholamines in the brain after sedatively and non-sedatively acting drugs can be seen in fig. 3 The above mentioned results are computed from the data of other experiments done under corresponding conditions The 5-HT levels are plotted against the catecholamine levels (NA + DA) Three groups of data are seen in this figure The levels of 5-HT after treatment with substances with no sedative effect in the doses administered (black squares) are not very different from the control values (white squares) although the catecholamines are clearly lowered. Reserpine and bishomoreserpine (white circles) present an apparently different group of data in that all monoamines are decreased

These results when correlated to the estimation of spontaneous motility show that the data in the upper part of fig. 3 represent experiments in which no decrease in motility was seen Monoamine levels concentrated in the left lower quadrant were associated with a striking decrease in spontaneous motility

Discussion

It might be tempting to draw the conclusion from the present data, that the sedative action of reserpine is related to 5-HT rather than the catecholamines reserpine analogues with little or no action on the brain 5-HT but with a clearcut effect on the catecholamines are found to be devoid of any sedative action However great caution should be exercised in drawing conclusions merely from amine levels without any further analysis of the mechanisms involved. It should be kept in mind that the major fraction of the monoamine stores does not seem to be essential for the function. In this connection it may be significant that even large doses of a compound such as mediodespidine do not seem capable of bringing the brain catecholamines down to the extremely low levels characteristic of the action of reserpine In other words, the monoamine depletion by mediodespidine may prove insufficient to bring about blockade of catecholamine and 5-HT transmission mechanisms in the brain. Whether this type of compound interferes with peripheral adrenergic transmission remains to be investigated. Such interference should lead to ptosis, which was not observed after mediodespidine treatment. This calls for further investigations on the mechanism of the hypotensive action of this agent.

Summary

The depletion of 5-HT and catecholamines from the mouse brain and heart after administration of five reserpoids is described. Derivatives lacking sedative and ptosis-producing effects, had little influence on the 5-HT level in the brain and reduced noradrenaline and dopamine levels to variable degrees they seemed unable to bring about the marked depletion characteristic of reserpine. Another analogue, with a reserpine-like sedative action, lowered the 5-HT as well as the catecholamine content in the brain. The results are discussed with regard to possible mechanisms of actions.

Acknowledgements

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Autoradiographic Distribution Studies of Adrenergic Blocking Agents. I ^{14}C Phenoxybenzamine (Bensylyt NFN), an α Receptor Type Blocking Agent*

By

David Masuoka Lars-Erik Appelgren and Eskil Hansson

(Received July 25 1966)

A dual adrenergic receptor mechanism has been proposed by AHLQUIST (1948) to classify receptors with regard to their sensitivity to sympathomimetic amines and the inhibition of their response to these drugs by certain adrenergic blocking agents. Thus dibenamine and related haloalkylamines have been shown to produce a potent and prolonged blocking action of the α receptors (NICKERSON & GOODMAN 1947 NICKERSON 1949), while agents such as dichloroisopropylarterenol (DCI) (POWELL & SLATER 1958) block only β-receptors.

In the present paper the distribution in mice of the α receptor blocker phenoxybenzamine (dibenzyline ®), has been studied by the whole-body autoradiography technique of ULLBERG (1954) in order to compare its uptake and binding characteristics by organs and tissues. In a subsequent paper the distribution of a β-receptor blocker propranolol, is described (MASUOKA & HANSSON 1967) It was hoped that such studies might provide further knowledge about adrenergic receptors and help to settle certain controversies regarding the pharmacology of blocking agents.

Methods

Radioactive Compound. ^{14}C-phenoxybenzamine hydrochloride labelled in the methylene carbon was obtained from the Smith Kline & French Laboratories, Philadelphia, U.S.A.

*) Aided by National Institutes of Health grant (MH-03663) and the Swedish Medical Research Council.

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The specific activity of the compound was 1.3 mc/mM The radiochemical purity of the compound was also checked immediately before injection, by thin-layer chromatography and exposure of the plates to X ray film as described below The autoradiogram revealed one major spot which corresponded to the R of phenoxybenzamine and represented 9 / of th total counts, and three minor spots.

A toradiography Two μc (0.54 mg) of ^{14}C-phenoxybenzamine-HCl in 0.2 ml acidified propylene glycol-physiological saline solution prepared as described by NICKERSON & GOODMAN (1947), were injected intravenously in the tail vein of male and female albino mice of the NMRI strain weighing 20–22 g. The mice were sacrificed 5, 20, and 40 minutes, 1 4 and 24 hours and 2 and 4 days after the injection. Under ether anesthesia, they ere immersed in hexane cooled with solid carbon dioxide. Whole body autoradiography was done according to the ULLBERG method (1954) The various steps involving sectioning, exposure of freeze-dried sections to osmic vapor and placing the sections against X-ray film were carried out in cold room at −10° Sections thirty μ thick were exposed to Structurix (Gevaert), and 80 μ thick sections to Kodirex (Kodak)

Collection of bile Two male rats of the Sprague Dawley strain (body weight 250 g) were anesthetized with pentobarbitone given intraperitoneally The abdomen was opened by incision and polyethylene tube inserted into the common bile duct. The rats were injected with 5 μc (1.35 mg) ^{14}C phenoxybenzamine-HCl administered into the femoral vein. The bile was collected in beakers for four hours.

Thin-layer chromatogr phy Thin-layer silica gel plates were prepared according to STAHL (1962) The solvent systems were chloroform-methanol (80 1) and heptane-chloroform-methanol (80 33 13). The radioactivity on the chromatogram was detected by ex posure to Kodirex X ray film, and the colour spot of uthentic phenoxybenzamine was developed with iodoplatinate reagent.

Tissue extraction. Two male mice (body weight 20 g) were injected intravenously ith 5 μc (1.35 mg) ^{14}C-phenoxybenzamine HCl. The animals were killed 40 minutes and 24 hours after the injection. The brain, heart, brown f t, liver and skeletal muscle ere removed from the mouse injected 40 minutes previously d the brain, heart and skeletal muscle from the mouse injected 24 hours previously Each tissue sample was homogenized in distilled water and adjusted to pH 3 by addition f 0.1 N HCl. The samples are extracted with 20 ml heptane. The radioactivity in the heptane, water phase and the tissue debris was determined by liquid scintillation counting. The volume of heptane was reduced by evaporation under nitrogen and the remaining heptane from each sample used for thin-l yer chromatography

Results

Whole-body autoradiography

With the autoradiographic method it is possible to trace the injected labelled compound and/or its metabolites in the various organs at dif ferent times after administration. After the injection of ^{14}C phenoxybenzamine, the radioactivity did not appear to leave the blood rapidly and up to 40 minutes was required before there was significant clearance. The tissues which contained the highest activity were the liver kidney and brown fat. The radioactivity penetrated readily into most tissues. The retention differed in various tissues, some tissues retaining activity

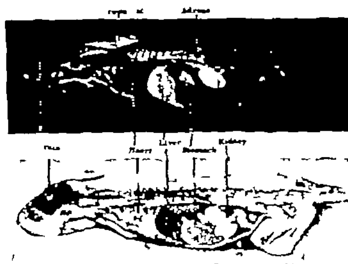


Fig. 1 Autoradiogram (upper photo) showing distributio f radioactivity (light areas) in mouse 40 minutes after intravenous injection of [14]C-phenoxybenzamine. Correspond ing osmium-treated tissue section (lower photo). 30 µ.

for long periods (fig. 1–4) The distribution in various organs is described in detail below

Central nervous system. Five to 20 minutes after the injection, the gray matter of the brain appeared to have about the same activity as the blood.

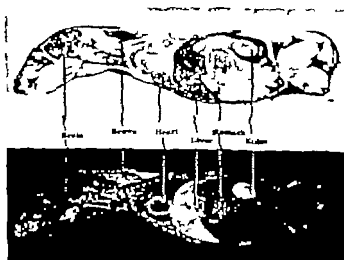


Fig 2. Autoradiogram (pper photo) showing distribution of radioactivity (light reas) in mouse 4 hours after intravenous injection f [14]C-phenoxybenzamine. Corresponding osmium-treated tissue section (lower photo) 30 µ.

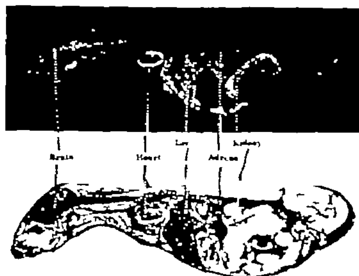


Fig 3 Autoradiogram (upper photo) showing distribution of radioactivity (light areas) in mouse 2 days after intravenous injection of ^{14}C-phenoxybenzamine. Corresponding osmium-treated tissue section (lower photo) 80 µ.

Then, as the radioactivity in the blood decreased, the activity in the CNS remained continuously higher than that in the blood during the remaining 4 day period of observation. Hence, although phenoxybenzamine did not appear to be actively concentrated in this tissue, having once pene-

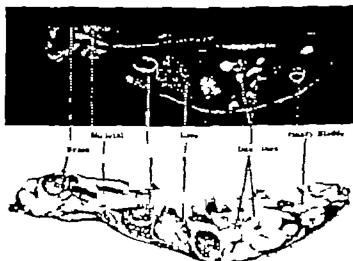


Fig 4 Autoradiogram (upper photo) showing distribution of radioactivity (light areas) in a mouse 4 days after intravenous injection of ^{14}C-phenoxybenzamine Corresponding osmium-treated section (lower photo) 80 µ.



Fig. 5 Enlargements of an autoradiogram (left) and corresponding tissue section (right) 20 minutes after intravenous injection of ^{14}C-phenoxybenzamine.

trated it did not appear to leave readily Distribution within the brain was fairly even.

Cardiovascular system Within 5 minutes the myocardium had a rather high activity which was retained for a long period, and in the 4-day animal, this tissue contained the highest radioactivity of any organ in the body (fig. 4)

The large blood vessels were as highly labelled as the heart still contained activity after 24 hours.

Endocrine glands The pituitary and pineal within 5 to 20 minutes after the injection, showed activity which was slightly higher than that in the blood and brain After 1 hour the pituitary was still slightly more radioactive than the brain.

The adrenal cortex (fig. 1) showed a high concentration within 5 minutes of the injection of ^{14}C-phenoxybenzamine. The activity was maintained above the blood level for the entire 4 day period. The activity in the medulla was never much higher than that in the blood (fig. 5)

Digestive system. Rather high activity appeared in the mucosa of the fundus and stomach content as early as 5 minutes after the injection. The intestinal walls, however did not accumulate radioactivity higher than that in the blood while their contents were seen to be very active at 40 minutes and still active at 4 days

Initially the salivary glands were found to be only as active as the blood concentration but they remained slightly higher after 4 hours.

The liver was highly labelled at 5 minutes and gradually decreased in activity but was still moderately active at 24 hours. The radioactivity of the bile was observed to be very high as early as 5 minutes and remained high up to 24 hours.

Lymphatic tissue The lymph nodes, thymus, and white pulp of the spleen did not accumulate radioactivity and had, in fact, a much lower activity than the blood for at least one hour

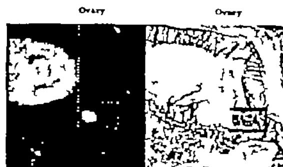


Fig. 6. Enlargements of an autoradiogram (left) and corresponding tissue section (right) 4 hours after intravenous injection of ^{14}C-phenoxybenzamine

Adipose tissue Brown fat was seen to be very intensely labelled in the 5 minute and only slightly less in the 1 hour – and 4 hour autoradiogram. However after 24 hours it had decreased significantly and the tissue no longer contained the highest radioactivity

White adipose tissue was continuously lower in radioactivity than the blood.

Skeletal muscle and bone The activity in skeletal muscle was about the same as that in the blood initially but after one hour it remained slightly higher throughout the remainder of the experimental period.

Hard bone was not penetrated by the ^{14}C label while activity in the bone marrow merely appeared to follow the blood level.

Ovary The corpus luteum (fig. 6) was observed to have high activity in one hour and was still high after 2 days. The follicles, however were not radioactive.

Bile excretion

The experiments with bile fistulae showed that in 4 hours 29.3 and 32.8 / of the radioactivity was excreted into the bile of the two rats investigated

When the bile was extracted with heptane only traces of radioactivity were extracted into the heptane layer Thin-layer chromatography of the bile revealed that the radioactivity in the bile did not move in the heptane-chloroform-methanol system, while the authentic phenoxybenzamine had an R_f-value of 0.8

Identification of radioactivity in tissues

Certain tissues were removed from two mice 40 minutes and 24 hours after injection of ^{14}C-phenoxybenzamine, extracted with heptane and the

Table 1

Percent of radioactivity in tissues which represent ^{4}C-phenoxybenzamine 40 minutes and 4 hours after i. injection in mice.

Tissue	% phenoxybenzamine (according to R_f-value)	
	40 minutes	4 hours
Brain	51	73
Brown f t	87	–
Heart	61	76
Liver	29	–
Skeletal muscle	71	81

extracts chromatographed as described under METHODS The brain, heart, liver and skeletal muscle from the 40 minute and 24 hour mice all showed radioactive areas corresponding to the R_F of authentic phenoxybenzamine Table 1 shows that a high percentage of the radioactivity remaining in the various tissues at 40 minutes and 24 hours is due to a compound having an R_F corresponding to that of phenoxybenzamine. Only the liver appeared to have a larger percentage of metabolized phenoxybenzamine.

The radioactivity from brown fat formed an unusual "U" -shaped spot (probably due to excess lipid) on the autoradiogram in the phenoxybenzamine area. In order to confirm that this was phenoxybenzamine, brown fat and brain from two mice were removed 40 minutes and 4 hours after injection of ^{14}C phenoxybenzamine. The heptane extract was evaporated to dryness under reduced pressure, and the residue extracted with acetone. An aliquot of the acetone extract was chromatographed. After exposure to X-ray film, the chromatogram plate was sprayed with iodoplatinate reagent. The dark spots on the autoradiogram had the same R_F as authentic phenoxybenzamine.

Discussion

The diffuse nature of the distribution of ^{14}C-phenoxybenzamine was not completely unexpected in view of the fact that this compound has been shown to react readily with sulfhydryl, amino, and carboxyl groups *in vitro* (HARVEY & NICKERSON 1954). The distribution of radioactivity may be summarized as follows

40 minutes to 1 hour Brown fat > kidney > liver gastric mucosa, adrenal cortex, heart, corpus luteum > blood

1 day Heart > corpus luteum certain skeletal muscles, adrenal cortex, kidney > blood

2 days Heart > corpus luteum, certain skeletal muscles, brain > liver adrenal cortex > kidney > blood.

4 days Heart > brain > certain skeletal muscles, liver > adrenal cortex, kidney > blood, brown fat.

Dibenamine and related haloalkylamines have been shown to possess a specific, potent, and prolonged adrenergic blocking action (NICKERSON & GOODMAN 1947) The mechanism of this action has been assumed to be due to a relatively stable combination of the blocking agent with a specific tissue receptor On the other hand, BRODIE *et al* (1954) have suggested that the prolonged pharmacological effect could be explained by the accumulation of phenoxybenzamine in fat depots from which it slowly diffuses into the blood stream. Following a moderately high dose of ^{14}C phenoxybenzamine, the present investigation has shown that a good deal of activity was initially localized in brown fat. Later (2–4 days) when the concentration in the brown fat had decreased to low levels, there was still radioactivity specifically bound in certain other tissues. Propranolol, 24 hours after the injection of the same amount of radioactivity *shows no such tissue binding under the same conditions of* exposure (MASUOKA & HANSSON 1967) It is, therefore, concluded in agreement with NICKERSON (1962) that because of persistent binding by certain tissues, accumulation in fat depots is not essential for the prolonged blocking activity of phenoxybenzamine. This does not exclude the possibility however that with high doses slow release from fat depots may extend the duration of activity

The marked accumulation of radioactivity in brown fat deserves further comment. The high lipid solubility property of phenoxybenzamine alone could not explain this accumulation, in as much as white adipose tissue did not significantly take up radioactivity In addition, HANSSON & SCHMITERLÖW (1961) have observed that a lipid insoluble drug aprobit ® (an antihistamine with some α blocking property) had a greater tendency to accumulate in brown fat than one which was more lipid soluble. Since the noradrenaline content, especially in rats (STOCK & WESTERMAN 1963) and the adrenergic innervation pattern (see WIRSEN 1965) of brown fat is markedly greater than that of white adipose tissue and since these drugs, both with α-receptor blocking action but with different lipid solubility properties, are concentrated *in brown fat, it is tempting* to assume that this high accumulation is related in some way to the receptor

The presence of α receptors in brown fat however still remains to be demonstrated

The present study has also shown that in all probability the intestinal contents exhibit very high labelling from 20 minutes to 4 days. The source of this high activity is the bile, since approximately 30 / of the total radioactivity was observed in the bile during the 4 hour collection period in rats.

The autoradiograms indicate that phenoxybenzamine is taken up quite rapidly by the heart and is tightly bound. In the 4 day picture, the heart shows the highest retention of activity among all the organs. Although the chronotropic and inotropic effects of catecholamines are not inhibited by α-blockers, the haloalkylamine blocking agents do prevent cardiac arrhythmias induced by sympathomimetic amines, especially after sensitization by cyclopropane and other hydrocarbons. Such a protective effect against arrhythmias, which has been said to persist for 24 hours to 8 days (WILBURNE *et al* 1947), is thus consistent with the specific, long-lasting radioactivity in the heart.

One of the major objections to whole-body autoradiography is the fact that the radioactivity in the tissues may not represent the labelled drug which was injected but may be due to metabolites. Therefore, some means of identifying the compound responsible for the radioactivity is necessary In the present experiment, thin-layer chromatography with appropriate carriers was used. The radioactivity bound in some tissues at 40 minutes, 4 hours, and 24 hours after intravenous injection is still mainly due to intact phenoxybenzamine or to a compound which, when extracted under the present conditions, has the same R_F as phenoxybenzamine. It has been postulated (NICKERSON *et al* 1946) that the pharmacological activity is due to the formation *in vivo* of a cyclic ethyleniminium ion If alkylation occurred on the receptor one would expect a radioactive spot possibly corresponding to the hydrolysis product, N phenoxyisopropyl N benzyl-β-hydroxyethylamine Perhaps the receptors in the organs analyzed represent only a very small fraction of the tissue mass and minor radioactive components in the thin-layer chromatogram should be closely scrutinized

Summary

The distribution of ^{14}C phenoxybenzamine has been studied in mice by the whole-body autoradiographic method. The radioactivity penetrated readily into most tissues. Although the liver kidney and brown fat initially contained the highest activity certain organs such as the heart and central nervous system retained higher activity after 4 days.

Certain tissues, therefore, appear to exhibit prolonged binding of phenoxybenzamine.

The radioactivity in various tissues after 40 minutes and 24 hours was identified by thin layer chromatography. Radioactivity spots having R_F values similar to that of authentic phenoxybenzamine were observed. If N-phenoxyisopropyl N-benzyl-β-hydroxyethylamine was present in the tissue extracts, it was not in a high percentage.

Acknowledgements

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Acta pharmacol et toxicol 1967 **25** 123–126

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A Simplified Method for Extracting Phenytoin from Serum and a More Sensitive Staining Reaction for Quantitative Determination by Thin-Layer Chromatography

By

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(Received June 14, 1966)

In a previous paper (OLESEN 1965) a method was described for deter mining phenytoin in serum in the presence of drugs which interfere with the spectrophotometric method (SVENSMARK & KRISTENSEN 1963)

Further experiments showed that for the determination of such small amounts of phenytoin, uniform Rf values for standards and test solutions could not be achieved, without purifying the residue left after evaporation from the chloroform extraction. In addition, the concentrated and un purified extract had a tendency to produce tailing, probably because of the plate was overloaded with strongly adsorbent substances at the starting point.

In the previous method, however the purification process was both time consuming and involved many manipulations a simplification has thus been worked out, and many hundreds of determinations of phenytoin have now shown that the process of purification can be considerably simplified.

Increased sensitivity of the staining reaction has also been found of help in the visual quantification.

Method

Amounts of serum of 3 ml and 1 ml, respectively are shaken vigorously with 0.1 ml f concentrated HCl and 25 ml of chloroform for about 10 seconds pproximately 10 g of anhydrous sodium sulphate is added, and the shaking repeated for about 10 seconds. The solution is then filtered int burettes with glass stopcocks and attached funnels. A olume of 21 ml of the chloroform filtrat is measured int 50 ml Erlenmeyer flasks.

The chloroform is ev porated on water bath at 60–70° by means of a stream of air

The residue on the water bath is treated with 3 ml of methanol with continuous stirring After cooling to room temperature the methanol extract is transferred to pointed centrifuge tubes, the flask being rinsed with 2 ml of cold methanol which is also added to the centrifuge tubes. The methanol is evaporated and the centrifuge tube rinsed with 2 ml of chloroform which is again evaporated to dryness.

Chromatography

The procedure of applying standards and test solutions is exactly as previously described (OLESEN 1965) the composition of the solutio for separating the constituents in the chromatogram has, however been altered, so that it now consists of chloroform, methanol and water in the proportions 75 25 1

Staining reactions

The treatment of the plate with piperidine nd chloroform in unchanged, but by reducing the distance to the source of U V radiation (Philips TUV 15W L4), the period of radiation is reduced to 5 minutes. The plates are then moistened with a 2% aqueous solution of $CuSO_4$ (previously 1%), and dried in a stream of cold air The phenytoin now appears as yellow spots. The plates are then moistened thoroughly with freshly prepared mixture of 1 N NaOH and 1% aqueous solution of 4-pyridinium chloride, in the proportional 1 1 and the phenytoin is stained reddish-orange, while any barbiturates present are stained the same colour

Results

Recovery and specificity

As a result of the alteration in the method of extraction recovery is slightly increased (approximately from 84 % to 87 %) In order to maintain the simple proportion between the phenytoin content of the standards and the sample, this has been compensated for by taking only 21 ml of the chloroform extract for the working procedure.

The staining reaction as originally described, still forms the basis for the qualitative identification of phenytoin The specificity of the staining reaction received further confirmation from results obtained in the qualitative demonstration of phenytoin in the faeces, brain, liver kidneys and the heart musculature of pigs poisoned with phenytoin In addition, no difficulty was encountered in carrying out a single qualitative demonstration of the presence of phenytoin in the gastric contents (see figure) from a patient who had taken the drug with suicidal intent.

The new staining reaction with 4-pyridyl-pyridinium chloride which is carried out after the qualitative demonstration is about 10 times as sensitive as the staining reaction with piperidine-copper sulphate but at the same time it is rather non-specific. It can best be compared with mercury sulphate-diphenyl-carbazone staining. It has been found of advantage however to be able to demonstrate barbiturates, for example,

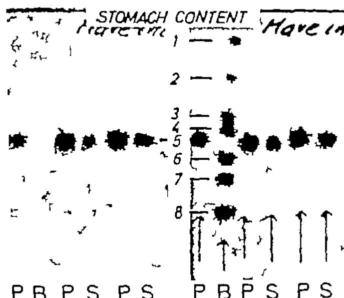


Fig 1 Identification of phenytoin extracted from stomach contents. The left side of the photograph illustrates the specific reaction with Cu^{++}-piperidine. The right half of the photograph shows the result of subsequent staining of the same plat with 4-pyridyl-pyridinium chloride, together with the position of the phenytoin in relation to glutethimid and some barbiturates. The extract from the stomach contents was applied at point P pure phenytoin was applied at point S and a barbiturate mixture at point B Spot no. 1 glutethimidum, 2 Enhexymalum, 3 Mebumalum, 4 Pentymalum, 5 Phenytoin, 6 Heptabarbitone (Brit.), 7 Diemalum, and 8 Phenemalum.

after the qualitative demonstration of the phenytoin. This more sensitive reaction has been found to be particularly valuable in the quantitative estimation of the phenytoin content.

Discussion

The treatment of the evaporated chloroform extract with methanol instead of water saves the cooling and the re-extraction with chloroform. This is a great saving, both in time and work. The extract is more contaminated, but the same advantage is obtained by transferring the phenytoin to the water phase, namely avoiding the substances which cause tailing on the plates. Some degree of distortion of the spots may be observed, but this disadvantage has been balanced by the increase in the sensitivity of the colour reaction.

When NaOH is added to 4-pyridyl-pyridinium chloride, the very labile glutaconic aldehyde and 4-amino-pyridine are formed. ANGER & OFRI

(1964) use glutaconic aldehyde as a coupling reagent, although hydrochloric acid must be added to bring the substance from the enol to the aldehyde form. If the plate is treated with hydrochloric acid after the staining reaction has been performed as described here, the spots disappear or become bleached, so that it is probably more a case of oxygenation than of coupling.

Summary

A simplified method is described for the extraction of phenytoin from serum, the residue from the evaporation of the original chloroform extraction being transferred to methanol instead of to water

A staining reaction with glutaconic aldehyde is also described, which is almost 10 times as sensitive as the piperidine-copper sulphate reaction it can be carried out on the thin layer plates following the specific qualitative test with piperidine and copper sulphate

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Actions of Prenylamine (Segontin ®) on the Cardiovascular System

By

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(Received May 26, 1966)

Prenylamine (*segontin* ®), *2-(3,3-diphenylpropylamino)-phenyl-propan,* depletes various tissues of their catecholamine stores (for references see OBIANWU 1967). It has also been reported to increase the coronary blood flow in the isolated guinea pig heart (LINDNER 1960) and to increase oxygen saturation of coronary sinus blood (BÖHN SCHLEPPER & WITZLER 1960 KOCHSIEK, BRETSCHNEIDER & SCHELER 1960) There are numerous reports of clinical trials on the value of prenylamine in the treatment of angina pectoris. For references see BAUMGARTEN (1962) and ABRAHAMSEN (1963)

The results of this investigation indicate that in various animal species prenylamine lowers the mean arterial blood pressure and the heart rate, antagonizes responses to tyramine and isoprenaline and potentiates those to noradrenaline (NA) and adrenaline (A). In rabbits and guinea pigs, the fall in blood pressure elicited by isoprenaline is reversed to a pressor response

Material and Methods

Blood pressure of anaesthetized cats and rabbits

6 cats, 1.8–3.5 kg in weight were anaesthetized with pentobarbitone sodium (mebumal sodium NFN = nembutal ®) 30 mg/kg body weight, given intraperitoneally 12 rabbits, 2–4 kg, were anaesthetized with pentobarbitone sodium 30 mg/kg and 25 % urethane 5 ml/kg by intraperitoneal injection. A tracheal cannula was inserted and artificial ventilation was administered when necessary The carotid (or femoral) artery was cannulated and connected to a Grass model P 23 Dc pressure transducer The blood pressure was recorded by means of a Grass model 5 polygraph. The heart rates were determined from tachograph recordings. Prenylamine was given   dose of 5–8 mg/kg. Drugs were usually injected into a jugular vein.

Blood pressure of anaesthetized rats and guinea-pigs

Rats, weighing 250–400 g, and guinea-pigs, weighing 350–800 g were anaesthetized with pentobarbitone sodium 30–36 mg/kg given intraperitoneally and the arterial blood pressure recorded as described above. In some of the experiments rats were pretreated with prenylamine 20 mg/kg intraperitoneally twice at 12 hrs. interval

Blood pressure of conscious rats and guinea-pigs

Rats and guinea-pigs were anaesthetized with pentobarbitone sodium and the aorta and vena cava permanently intubated, using polyethylene tubing as described by POPOVIC & POPOVIC, (1960). When the animals had fully recovered (usually in 4–5 days) the blood pressure was recorded for at least 3 hrs. on each of the two successive days. Prenylamine was given intraperitoneally in two doses at 12 hrs. interval and the blood pressure recorded for several hrs. each day for about four days.

Myocardial contractile force and rate in cats

Four cats, weighing 2.5–3.5 kg, were anaesthetized with pentobarbitone sodium 30 mg/kg body weight. The chest was opened through a midsternal incision while respiration was maintained with a positive pressure pump. A strain gauge arch¹) was sutured to the right ventricle and the myocardial contractile force was recorded on a Grass polygraph (model 5). Arterial blood pressure was measured as described above. By running the chart at a suitable speed (usually 5 mm/sec.) the heart rate was directly recorded.

Bilateral cervical vagotomy was performed in all experiments. Body temperature was maintained at 37° with an infrared lamp, thermostatically regulated by a thermometer inserted in the rectum. Drugs were injected into the jugular vein. Prenylamine in a concentration of 2 mg/ml was usually infused into a femoral vein at the rate of 0.5 ml/minute by means of an adjustable constant infusion apparatus (B. BRAUN, Melsungen, Germany).

Isolated perfused heart

Rabbits were stunned and bled and the hearts perfused according to LANGENDORFF with McEWEN's solution (1956) at 37° aerated with carbogen (O_2 95 % and CO_2 5 %). The contractions were recorded semi-isometrically by means of force displacement transducer Grass model FT 03 and Grass model 5 polygraph. In some of the experiments the recordings were made as described by ANDÉN, CORRODI, ETTLES & GUSTAFSSON (1964).

Rabbit isolated atria

Rabbit atria were isolated and suspended in 50 ml organ bath at 37°. The bath fluid and recording were as for the isolated heart.

Results

Effect of prenylamine on blood pressure of anaesthetized cats, rabbits, rats and guinea-pigs

The intravenous injection of 6 mg/kg of prenylamine (cats and rabbits) and of 10 mg/kg (rats and guinea-pigs) caused an immediate and sharp

¹) The strain gauge arch is the type used at the Research laboratories of AB Hässle, Göteborg, Sweden, and was kindly supplied by Mr Olof Gustafsson

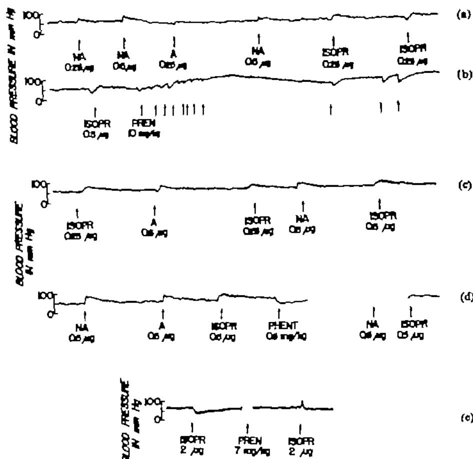


Fig. 1 Blood pressure f guinea-pig weigh g 800 gm anaesthetized with pentobarbitone sodium 35 mg/kg p.

( ) Indicates response to doses of noradrenaline (NA), adrenaline (A) nd isoprenaline (ISOPR) NA & A were calculated as the base and isoprenaline as the salt.

(b) Shows the transient falls in blood pressure following injections of prenylamine (PREN) 10 mg/kg i. (total dose injected). Arrows indicate injections of prenylamine. Note slight rise in blood pressure which was not sustained.

(c) Show the responses t NA, A and isoprenaline after PREN Note the pressor response to isoprenaline.

(d) Show that the dose of phentolamine (PHENT) which bolished the responses to NA and A also antagonized the pressor response to isoprenaline.

(e) Blood pressure recording of a rabbit weigh g 2.5 kg anaesthetized with mixture of pentobarbitone sod m 30 mg/kg nd 5 ml/kg of 25% solution of Urethane. At the rrow prenylamine 7 mg/kg was given intravenously About 20 minutes later ISOPR was given.

Table 1

Effect of prenylamine on arterial pressure and heart rate in cat.

	Before prenylamine	After prenylamine 6 mg/kg i.v.
Arterial pressure in mm Hg	156 ± 8 (6)	No change
Heart rate/min.	225 ± 15 (6)	171 ± 9

Figures in brackets indicate number of experiments.

fall in arterial blood pressure which lasted for 2–3 minutes (fig. 1a). Usually prenylamine was injected slowly over 10–15 minutes and the fall in blood pressure was observed each time an injection was given. When a slow continuous infusion of prenylamine was made with a constant

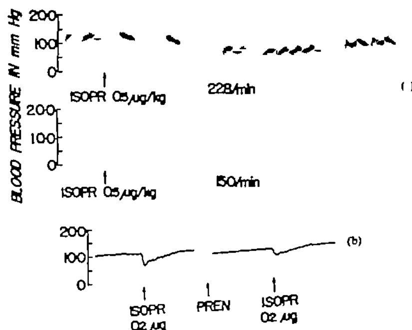


Fig. 2.

( ) Blood pressure recording of an open chest cat weighing 3 4 kg anaesthetized with pentobarbitone sodium 30 mg/kg.
The figure shows the response to isoprenaline (ISOPR) before prenylamine (top) and after prenylamine 6 mg/kg i. (bottom).
The numbers indicate the heart rates.

(b) Blood pressure of rat weighing 300 gm anaesthetized with pentobarbitone sodium 35 mg/kg.
It indicates responses to isoprenaline before (left) and after (right) prenylamine 10 mg/kg i.

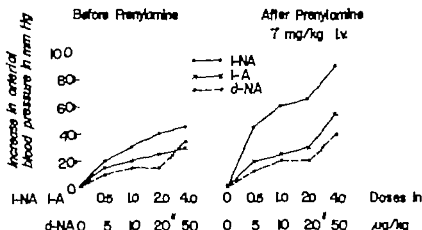


Fig. 3. Graph of pressor responses to isomers of NA and A before (left) and after (right) prenylamine in cats.
Note that the increase in sensitivity of the amines follows the order L NA > L A > D-NA.

infusion apparatus, there was a decrease in the blood pressure which lasted throughout the infusion period but the pressure usually returned to the pre-infusion level within a few minutes. After the infusion of prenylamine in most of the experiments in rabbits, guinea pigs and rats there was usually a slight rise in blood pressure, possibly due to release *of catecholamine from the binding sites (fig. 1 b)*

In anaesthetized cats, rabbits and guinea-pigs prenylamine reduced the heart rates by up to 25 / See below and table 1 Due to technical difficulties, the heart rates of rats could not be recorded.

In none of the anaesthetized animals was a fall in blood pressure observed during the experiments, each of which lasted for at least 6 hrs (apart from the transient fall following intravenous injections of prenyl amine).

The fall in blood pressure following injection of isoprenaline was antagonized by prenylamine in cats and rats (figs. 2a & b) and reversed to a pressor response in rabbits and guinea pigs (figs. 1c & e). The pressor response to injected NA and A were potentiated in cats, rabbits, guinea pigs and rats.

This potentiation seemed to be related to the degree to which the in jected amines were taken up at specific binding sites. This was tested in three cats by comparing the degree of potentiation of graded doses of L NA, L A and D-A. The result indicates that the potentiation is stereospecific and follows the order L NA > L A > D-NA (fig. 3)

Table 2

Effect of prenylamine o arterial pressure and response to tyramine in unanaesthetized rats nd guinea-pigs.

	Rat		Guinea-pig	
	Before	After prenylamine 40 mg/kg in two doses	Before	After prenylamine 40 mg/kg in two doses
Arterial pressure in mm Hg	122 ± 5	55 ± 7	75 ± 10	50 ± 6
Pressure response to tyramine in mm Hg	63 ± 5	23 ± 1		

Response to tyramine was not clear cut in the guinea-pig nd was therefore not included.

Blood pressure of unanaesthetized rats and guinea-pigs

The mean arterial blood pressures of unanaesthetized rats and guinea pigs fell by about 50 / and 25 / respectively (table 2) after two doses of prenylamine 20 mg/kg each at 12 hrs. interval. The pressor responses to tyramine (0 5 mg/kg) were reduced by about 70 / in rats. The responses in guinea pigs were not clear cut and were therefore not included.

Mycordial contractile force and rate in situ

This preparation offered an opportunity of observing the effects of prenylamine on the contractile force and rate of the heart and the blood pressure at the same time Infusion of prenylamine 6 mg/kg given intra venously reduced both the heart rate and amplitude of contraction (table 1)

This effect was observed as early as ten minutes after the start of the infusion and lasted up to 5 hrs after the infusion

The positive inotropic and chronotropic responses to isoprenaline were antagonized by prenylamine while those to NA were potentiated (fig 4) In the doses of A used the responses to A at least were not antagonized.

Isolated perfused heart

The results were qualitative similar to the in situ experiments. The response to isoprenaline was antagonized while that to NA was potentiated after slow infusion of prenylamine into the perfusion fluid just before entering the heart.

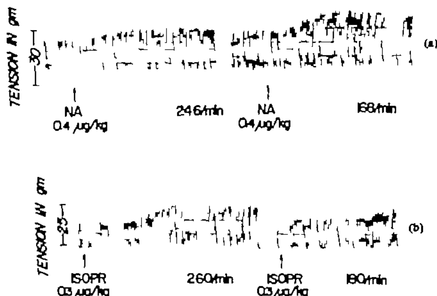


Fig. 4. Polygraph record of the contractile force and rate of the right ventricle of cat in situ.

(a) shows the response to NA before (left) and after prenylamine (right) 6 mg/kg I.

(b) shows response to isoprenaline (ISOPR) before (left) and after prenylamine (right) in the same cat.

Note that the response to NA was potentiated while that to ISOPR was antagonised by prenylamine. The numbers indicate the rates of contractions and the chart speed was 5 mm/sec.

Isolated rabbit atria

Prenylamine in a concentration of 6 µg/ml abolished the response to both isoprenaline and NA.

Pressor response to isoprenaline in rabbits and guinea-pigs

Prenylamine constantly reversed the fall in blood pressure produced by isoprenaline to a pressor response in the rabbit and guinea-pig. To test whether this response was due to activation of α-receptors, the pressor response to A and NA was blocked with phentolamine. From fig. 1d it would appear that the dose of phentolamine which blocked the pressor response to NA and A also antagonized the pressor response to isoprenaline. This suggests that the pressor response to isoprenaline after prenylamine is at least in part due to the activation of α-receptors.

Discussion

The failure of prenylamine to produce an effect on the blood pressure in anasthetized animals is surprising particularly since some of the animals (rats) were pre-treated with the drug. In view of the fact that there was a fall in blood pressure of about 50 / in unanaesthetized rats it seems likely that anaesthetic agents interfere with the actions of prenylamine. There are reports showing that the presence of anaesthesia may obscure the response to certain drugs (MAXWELL, PLUMMER, ROSS, DANIEL & SCHNEIDER, 1958). Several reports from clinical trials of prenylamine (see introduction for references) indicate that prenylamine slightly lowers the blood pressure and heart rate in man.

The present findings are consistent with the above reports though the more marked effects observed in animals could be due to the higher doses used. The doses used in clinical trials varied from 90 mg–180 mg/day as compared with the doses of 40 mg/kg used in rats.

The potentiation of injected NA and A after prenylamine observed in both anaesthetized and unanaesthetized animals is specific (see below).

After prenylamine, the pressor response to tyramine was greatly depressed (table 2) This is consistent with the finding that after prenylamine in doses of 180 mg/day for five days, the pressor response to tyramine in man is reduced by about 75 / (KUSCHKE, IDRIS & ECKMAN, 1965) This compares favourably with similar findings after small doses of reserpine in man (ABBOUD & Eckstein 1964)

There is no definite information on the response to NA and A after prenylamine in the report of KUSCHKE *et al* (1965) Such information is highly desirable in view of the clinical implications.

The depression of the pressor responses to tyramine after prenylamine could not have been due to a decrease in sensitivity of the vascular system since the responses to A and NA were potentiated and it is known that tyramine exerts its action by releasing NA from the binding sites (CARLSSON ROSENGREN BERTLER & NILSSON 1957 BURN & RAND 1958). A more likely explanation is a reduction in the NA content of the tyramine releasable amine pool It is conceivable that the decrease in the response to tyramine would have been greater if prenylamine had not potentiated the action of NA released by tyramine.

The study of the effect of prenylamine on the response to NA, A and isoprenaline in the cat heart in situ has provided further evidence that the potentiation of the response to NA and A is specific.

The positive inotropic and chronotropic effects of NA, A and isoprenaline are generally believed to be due to the activation by these amines of β-receptors in the heart. The uptake of NA at binding sites is

an important factor in the termination of its actions so that when this process is inhibited, the actions of NA are potentiated. Isoprenaline is normally not taken up at the storage sites in any appreciable amount (ANDÉN, CORRODI, ETTLES & GUSTAFSSON 1964. HERTTING 1964) and therefore is not potentiated when the uptake is inhibited.

The storage sites have a higher affinity for NA than for A and the relative rates of uptake of the stereoisomers of NA and A are L NA > L A > D-NA > D-A (WHITBY, AXELROD & WEIL MALHERBE 1961. IVERSEN & WHITBY 1962. MAICKEL, BEAVEN & BRODIE 1963).

Prenylamine potentiates the actions of NA more than that of A and antagonizes those of isoprenaline because of its moderate β-receptor blocking actions (see below). (Figs. 2 & 6). This potentiation is stereo-specific and follows the order L NA > L A > D-NA (fig. 4). These findings are consistent with the view that the potentiation of the actions of NA and A after prenylamine is due to inhibition of the uptake process of these amines at specific binding sites.

The uptake by the nerve terminals is a two step process: 1) Transport into the nerve terminals – the so-called "membrane pump" – and 2) subsequent incorporation into the amine storage granules. Substances that inhibit the first step (e.g. desipramine) cause potentiation of NA.

At present there is some evidence that prenylamine inhibits this step to a certain extent, CARLSSON & WALDECK (1965, 1966). The possibility that under the conditions of the present investigation prenylamine may have interfered with amine inactivation by the adrenergic nerves deserves further investigation.

MACKENNA (1965) showed that after rabbit hearts have been depleted of their NA content to 90 / by prenylamine, administration of large doses of NA is able to restore the NA content of the heart. In view of the very large doses of NA used in his investigation, the repletion might represent uptake at unspecific sites.

In an earlier experiment, OBIANWU (1967) found that pretreatment of rabbits with prenylamine abolished the response elicited by stimulation of periarterial nerves to the intestine. The addition of dopamine or NA to the bath fluid restored the response indicating that prenylamine does not prevent the uptake of these amines into the nerve terminals.

These observations may indicate that the inhibition of uptake by prenylamine could occur at sites outside the neurons. This view is supported by the findings of GILLESPIE & KIRPEKAR (1965) that after NA is infused into the splenchic artery various procedures including degeneration of the post-ganglionic splenic nerves, administration of cocaine and of adrenergic receptor blocking agents increased by about 60 / the recovery of NA which appeared in the venous blood. They explained their observation by

suggesting that tissue receptors play an essential role in the re-incorporation of NA into the nerve endings by acting as a "brake" on the diffusion away from the neighbourhood of the nerve terminals. In the absence of such a mechanism rapid diffusion away from the site of release of NA will limit the amount of NA (or time) available for re-incorporation. By combining with the receptors when released and then becoming dissociated from the receptors when the concentration in the synaptic cleft has been lowered by local diffusion some NA will be maintained for re-incorporation. Blockade of some of the receptors will inhibit this barrier to diffusion and consequently reduce the amount of NA re-incorporated.

Prenylamine has been shown to possess both α- and β-receptor blocking actions and may therefore inhibit this barrier mechanism. This increases the concentration of NA reaching the target organs.

In the isolated rabbit atria where prenylamine presumably is able to penetrate into the tissue more effectively than in the in vivo or in isolated heart experiments, both the actions of NA and isoprenaline are antagonized.

These observations allow the conclusion that the α- and β-receptor blocking actions of prenylamine are only observed in vitro and that in vivo only a moderate β-blocking action is important.

Reduction in sympathetic tone is unlikely to be the major mechanism involved in the lowering of blood pressure observed in unanaesthetized rats and guinea pigs. Prenylamine has only a weak sympathetic neurone blocking action in vivo and further the blood pressure is still depressed at a time when the amine levels have returned to normal (OBIANWU 1965).

Peripheral vasodilatation, lowering of the heart rate and the consequent reduction of cardiac output are more likely to be the major mechanisms involved in the lowering of blood pressure.

The mechanism of action of prenylamine in the reported relief of angina pectoris has so far not been elucidated. The suggestion that this is mainly due to coronary dilatation produced by prenylamine is unlikely particularly in old people with rigid and narrow coronary arteries. Coronary dilatation per se is of minor therapeutic importance in the relief of angina pectoris.

Marked increases in the blood catecholamine level have been observed in patients with angina pectoris immediately after exercise, during emotional excitement and during attacks of anginal pain (for references see RAAB 1962). Acute influxes of NA and/or A into the myocardium (e.g. during exercise, emotional excitement) intensify cardiac oxygen consumption and the resulting myocardial hypoxia is believed to cause pain (RAAB 1960). Recent clinical studies have demonstrated that β-adrenergic

blocking agents, pronethalol (nethalide) and propranolol (inderal) have beneficial effects in anginal patients (DORNHORST & ROBINSON 1962 HAMER, GRANDJEAN, MELENDEZ & SOWTON 1964 ALLEYNE *et al* 1963) and support the view that catecholamines play a part in the development of angina.

Prenylamine has been shown to possess a moderate β-adrenergic blocking action (the present investigation LINDNER 1964) and thus may largely account for its beneficial affect in anginal patients.

The heart appears to be very sensitive to the actions of prenylamine (OBIANWU 1965) so that a direct effect on myocardial metabolism and enzyme systems resulting in the same effect should not be ruled out

In view of the fact that the cardiovascular effects of NA and A are potentiated by prenylamine in vivo, combined therapy with β-receptor blocking agents may improve the efficacy of prenylamine in the relief of angina pectoris.

The lowering of blood pressure by prenylamine may make it unsuitable for hypotensive patients.

The observation that prenylamine reverses the fall in blood pressure elicited by isoprenaline in rabbits and guinea pigs is another indication of a β-receptor blocking action of prenylamine, as all previous observations of the pressor response to isoprenaline (see below) were observed after its adrenergic β-action had been blocked.

A pressor response to isoprenaline has been reported after pronethalol in guinea-pigs (SEKIYA & VAUGHAN WILLIAMS 1965) and after pronethalol and large priming doses of isoprenaline in cats (BUTTERWORTH 1963)

The present observation is consistent with the findings of SEKIYA & VAUGHAN WILLIAMS (9165) and extends the findings to rabbits.

BUTTERWORTH (1963) concluded that in cats, the pressor response to isoprenaline after priming doses of isoprenaline or pronethalol were not obtained with doses of the order of micrograms and that the pressor response to larger doses of isoprenaline was due to activation of α receptors.

SEKIYA & VAUGHAN WILLIAMS (1965), however disagreed on the ground that in their investigation, the pressor response to isoprenaline persisted when the pressor response to NA and A had been reduced to 1/5th by phentolamine.

Our findings is that the pressor response to isoprenaline is greatly reduced when the response to NA and A has been abolished by phentolamine (fig. 1 d) This may indicate that the pressor response to isoprenaline is at least partly due to the activation of the α-receptors.

SEKIYA & VAUGHAN WILLIAMS (1965) used a dose of isoprenaline (15 μg/kg) which may have been in excess of that required to produce a

maximum pressor response. In our investigation a much smaller dose of isoprenaline (0 6 μg/kg) was used This may explain the discrepancy between the two findings

Summary

The cardiovascular actions of prenylamine have been investigated on the blood pressure of anaesthetized and unanaesthetized rats and guinea-pigs, on the blood pressure of anaesthetized cats and rabbits, on the cat heart in situ and on isolated perfused rabbit heart and isolated rabbit atria.

Prenylamine lowers arterial pressure in *unanaesthetized* animals but fails to do so in anaesthetized animals.

Prenylamine lowers the heart rate

The fall in blood pressure, and the positive inotropic and chronotropic responses to isoprenaline are antagonized by prenylamine while the response to noradrenaline is potentiated.

The pressor response to isoprenaline (in rabbits and guinea pigs) after prenylamine is antagonized by phentolamine

The possible mechanism of action of prenylamine is discussed.

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Sympathetic and Receptor Blockade After Prenylamine (Segontin ®)

By

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Prenylamine (Segontin ® = 2-(3,3-diphenylpropylamino)-phenylpropane) has been shown to lower the amine levels of various tissues (SCHÖNE & LINDNER 1960 JUORIO & VOGT 1965 MACKENNA 1965 GROBECKER, PALM & SCHÜMAN 1965 OBIANWU 1965) and to inhibit the in vitro uptake of catecholamine by the storage granules of the adrenal medulla (CARLSSON, HILLARP & WALDECK 1963 EULER, STJÄRNE & LISHAJKO 1964 LUNDBORG 1966), of adrenergic nerves (EULER, STJÄRNE & LISHAJKO 1964) and the retention of amines by the heart in vivo (CARLSSON & WALDECK 1965) ANDÉN, MAGNUSSON & WALDECK (1964) provided evidence for a correlation between noradrenaline uptake and adrenergic nerve function after reserpine. Since many actions of prenylamine (including the inhibition of uptake by amine granules) are similar to those of reserpine it was considered of interest to see whether prenylamine had an adrenergic-neurone blocking action

Material and Methods

Unless otherwise stated, all recordings were made by means of Grass model 5 polygraph. Noradrenaline (NA) was determined by the methods of BERTLER, CARLSSON & ROSENGREN (1958) and HÄGGENDAL (1963).

5% solution of prenylamine gluconate was diluted with 5% glucose to give the appropriate concentrations. All concentrations are expressed in terms of their bases.

Cat nictitating membrane

6 cats, weighing 2–4 kg, were anaesthetized with pentobarbitone sodium (mebumal NFN = nembutal) 30 mg/kg body weight by intraperitoneal injection.

The contractions of the nictitating membranes in response to electrical stimulation were recorded semi-isometrically by means of Grass model FT 03 force displacement transducers. The cervical sympathetic trunk was prepared and divided preganglionically The cranial

stump was stimulated by square wave pulses using a Grass model SG 4 stimulator The blood pressure was usually recorded from the left femoral artery by means of Statham model p 23 Dc pressure transducer and drugs were injected into a jugular vein.

Rabbit nictit ting membrane

12 rabbits, weighing 2.3–3.5 kg, were used. They were anaesthetized with pentobarbitone sodium 30 mg/kg and urethane 5 ml of a 25 / solution/kg both administered intraperitoneally The nictitating membrane was prepared essentially as described by JACOBOWITZ, JOHNSON, KITCHENER & KOELLE (1963). A tracheal cannula was inserted and artificial ventilation administered with positive pressure pump when necessary The cervical sympathetic trunk was exposed and divided preganglionically The cranial stump was placed on a bipolar platinium electrode and covered with liquid paraffin. In two of the rabbits the electrode was placed o the superior cervical ganglion. Cervical vagotomy was carried out in all the experiments.

The cornea was incised with a scapel and the lens nd vitreous humor were expelled in order to deflate the eye. The eyelids were retracted and sewn back and the head was fixed rigidly by securing the lower and upper jaws between a groove o a rigidly fixed bar The stimulation was done as described above, at various frequencies (see results) 2 msec. duration and with a voltage that caused maximal contractio of the membrane. The recordings were as for the cat.

Contraction of the lower eyelid of th rat

Rats, weighing 250–300 g, were anaesthetized with either pentobarbitone sodium (34 mg/kg) or urethane (1.5 g/kg) given intraperitoneally A tracheal cannula was inserted and a jugular vein cannulated with a polyethylene t bing. The right cervical sympathetic chain was carefully isolated and placed on a bipolar pl tinium hook-electrode. Both vagi were cut nd supramaximal electrical stimulation at various frequencies (1 6, 3.2, 6.4, 12.8 and 25.6/sec.) with a pulse width of 0.5 msec. was applied for 10 sec. The retraction of the lower eyelid in response to electrical stimulation was recorded by means of a thread tied through the eyelid, passed through pulley and attached to a Grass model FT 03 force displacement transducer The recordings were made semi-isometrically o a Grass model 5 polygraph.

Two of the rats were pretreated with prenylamine 20 mg/kg twice daily for 2 days and were anaesthetized 2 hrs. after the last dose.

Isolated tissues

Segments of rabbit isolated intestine with their sympathetic nerves intact were prepared by the method of FIN LEMAN (1930). They were set up in 50 ml organ baths, in Mc EWEN's fluid (1956) at 37° and aerated with 95 oxygen nd 5% carbon dioxide. The periarterial sympathetic nerves were stimulated for 20 sec. every 3 minutes t various frequencies (10–50/sec) by means of Grass Stimulator SG 4 connected to an automatic device. I some experiments, lternat sections of ileum were set up under simila conditions and either prenylami e added to the bath nd stimulation continued until complete block was obtained, or stimulation without prenylamine continued for the same length of time. Tissues were removed from the bath, dried on filter paper weighed nd nalysed for noradrenaline.

Guinea pig vas deferens

Guinea pig vas deferentia were set up according to Hukovic (1961) and the hypogastric nerves stimulated for 20 sec. every 3 minutes at 30/sec. The bath temperature was 32° nd the responses were recorded by means of isotonic frontal writing lever on a smoked drum. Tissues were analysed for noradrenaline as above.

Results

Cat nictitating membrane

No effect on the contraction of the cat nictitating membrane elicited by the stimulation of the cervival sympathetic trunk was observed after various doses of prenylamine (6–10 mg/kg intravenously) during anaesthesia.

Pretreatment of the cats with prenylamine 15 mg/kg subcutaneously twice daily for 2 days (two cats) did not make any difference. The dose and duration of the treatment was limited by the high incidence of con vulsion which proved fatal in two cats.

The response to injected noradrenaline was potentiated. Potentiation to released noradrenaline may have compensated for any decrease in sympathetic tone.

Rabbit nictitating membrane

Histological and pharmacological evidence of the adrenergic nature of the sympathetic innervation of the rabbit nictitating membrane has been

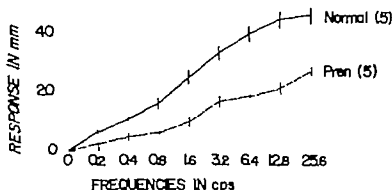


Fig. 1 Effect of prenylamine on semi-isometric contraction of the rabbit nictitating membrane in response t pre-ga glionic stimulation of the cervical sympathetic trunk. The parameters of stimulation ere 6 V 2 msec. for 30 sec. t the frequencies (shocks/sec.) indicated. Rabbits were pretreated with prenylamine (8 mg/kg I.V.) in two doses and anaesthetized 1 hr after the last dose. The interval between the first and second dose being 30 minutes. Vertical lines indicate standard errors of the means. — 5

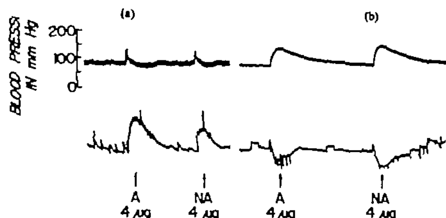


Fig. 2. Effect of prenylamine on the responses of the nictitating membrane and blood pressure of the rabbit to injected noradrenaline (NA) and adrenaline (A).
Rabbit 2.3 kg was anaesthetized with a mixture of pentobarbitone sodium (30 mg/kg) and 25% urethane (5 ml/kg) intraperitoneally (a) Before and (b) 30 minutes after prenylamine 7 mg/kg i.v.

provided (JACOBOWITZ & KOELLE 1964 JACOBOWITZ, JOHNSON, KITCHNER & KOELLE 1965) This is supported by the finding in the present investigation, that after reserpine treatment (5 mg/kg intravenously) the membrane failed to respond to nerve stimulation and that phenoxybenzamine (2 mg/kg intravenously) antagonized the response to stimulation. Pretreatment of rabbits with prenylamine (8 mg/kg intravenously in two doses) two hrs. before the experiment or 20 mg/kg subcutaneously twice daily for 3 days, reduced the response at all frequencies of stimulation.

The results are shown graphically in fig. 1 Administration of prenylamine (8 mg/kg intravenously) during anaesthesia did not give a clear cut effect. In 10 out of 12 experiments, after prenylamine treatment (whether before or during anaesthesia), injected noradrenaline and adrenaline caused a relaxation of the nictitating membrane while the pressor response was markedly potentiated A typical result is given in fig. 2.

A similar relaxation response to noradrenaline and adrenaline was observed in rabbits pretreated with reserpine (two experiments) This response was not influenced by an α receptor blocking agent (phentolamine 5 mg/kg intravenously) or by a new potent β-receptor blocking agent (56/28 – BROGÅRD EK & ÅBLAD personal communication).

Contraction of the lower eyelid of the rat

Preganglionic stimulation of the cervical sympathetic chain elicited retraction of the lower eyelid. This response was shown to be essentially sympathetic in nature (GERTNER 1956 SPRIGGS 1966)

In preliminary experiments this response was abolished by guanethidine (15 mg/subcutaneously) Prenylamine did not antagonize this response even in rats pretreated twice (20 mg/kg intraperitoneally) daily for 2 days

Isolated tissues

Rabbit isolated intestine

This preparation offered opportinities for using segments of intestine from the same rabbit in different investigations. Provided the gut was cleaned soon after being taken out, kept in cold (room temp) fluid through which carbogen (95 / oxygen + 5 / CO_2) was being bubbled, it could be used up to at least 6 hrs

Stimulation of the sympathetic periarterial nerves to the intestine caused relaxation. This response was completely abolished by prenylamine in quite small concentrations. The onset of blockade depended on the concentration of prenylamine in the bath. This varied from 30 min. (for 0 4 µg/ml) to 5 min. (for 5µg/ml) A typical result is shown in fig 3

Concentrations of prenylamine in excess of 0 5 µg/ml cuased a gradual progressive loss of rhythmic activity and tone, the rate of development being dependent on the concentration of prenylamine in the bath. This effect could be reversed by changing the bath fluid several times and was consistently prevented by the previous addition of phentolamine to the bath (see fig. 4) (GILLESPIE & MACKENNA 1961 observed a similar effect after adding reserpine to the bath fluid in which a piece of intestine was suspended but concluded that it was not specific)

Pretreatment of rabbits with 6 mg/kg prenylamine intravenously 3 hrs.



Fig. 3 Response of segment of intestine from a normal rabbit t stimulation of the periarterial sympathetic nerve.
Prenylamine (PREN) 0 4 µg/ml was given at PREN nd 20 minutes later the response was ntagonized.
Stimulation (dots) parameters. 3 2 msec. duration for 20 sec. at 40 shocks/sec. every 3 mi utes by means of an automatic device.

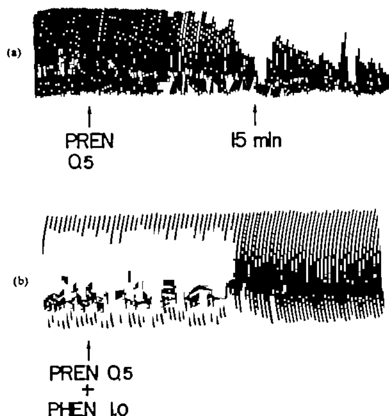


Fig. 4

( ) Isolated normal i testine of rabbit showing the effect f prenylamine on the rythmic activity nd tone (upper trace).

(b) An alternate segment of intestine to ( ). The prevention of loss of rythmic activity and tone (fter prenylamine) by phentolamine (lower trace).

The concentrations are µg/ml

before the experiment also abolished the response to stimulation of the periarterial nerves while the addition of noradrenaline or dopamine to the bath partially reversed the blockade. Whether prenylamine was added to the bath or the segment of the intestine was taken from a prenylamine treated rabbit, the blockade to stimulation of the periarterial nerves could not be reversed by changing the bath fluid several times.

Prenylamine in a concentration which when added to the bath blocked the response to sympathetic stimulation also markedly antagonized the response to added adrenaline, noradrenaline and isoprenaline This antagonism seemed to be reversible since it was overcome by higher concentrations of the sympathomimetic amines (fig. 5c) When the blockade to added sympathomimetic amines was completely reversed (by increasing

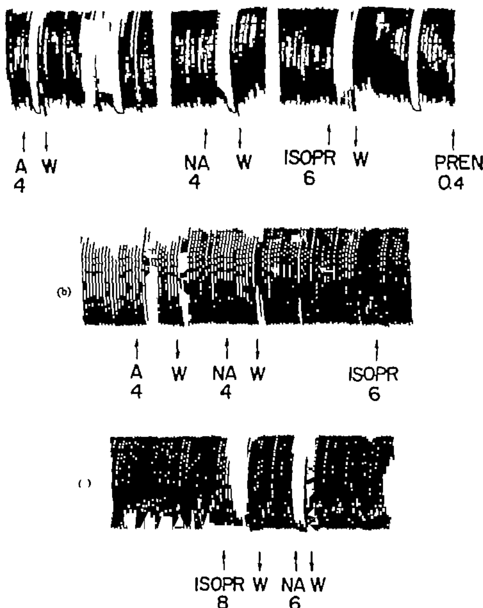


Fig. 5. Response of isolated normal rabbit intestine to sympathetic stimulation (dots), adrenaline (A), noradrenaline (NA) nd isoprenaline (ISOPR). Before prenylamine (PREN) ( ), after prenylamine (b), (c) shows reversal of the ntagonism to responses of the amines by increasing their concentrations. The concentrations re given in μg/ml and at w the bath was washed. Note that in (c) when the block to noradrenaline nd adrenaline was reversed, block to sympathetic stimulation still persisted.

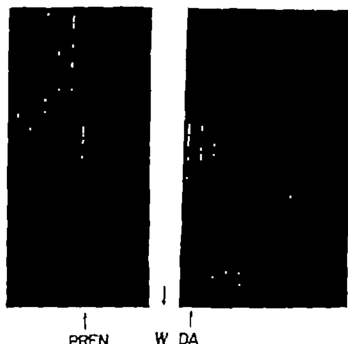


Fig. 6. C ntraction of vas deferens isolated from a normal guinea pig in response to preganglionic stimulation of the hypogastric nerve. Block of the sympathetic stimulation by prenylamine (5 µg/ml and partial reversal by addition of dopamine (10 µg/ml). Parameters of stimulatio 2 msec t 30/sec. for 20 sec. every 3 minutes. At w the bath was washed

the concentration of the amines) the blockade to sympathetic nerve stimulation still persisted (fig. 5c) In this way it was possible to separate the two independent blocking actions of prenylamine.

Guinea pig vas deferens

Prenylamine in concentrations of 1 to 5 µg/ml consistently abolished the responses of the guinea pig vas deferentia to preganglionic (see BIRMINGHAM & WILSON 1963) stimulation of the hypogastric nerve. The onset of the blockade was rapid and could not be reversed by changing the bath fluid but could be partly reversed by addition of dopamine (10 µg/ml) or noradrenaline (1 µg/ml) (See fig 6)

The response of the vas deferens from guinea pigs pretreated with prenylamine (30 mg/kg intraperitoneally) 3 hrs. before the experiment was greatly reduced but was partially reversed by the addition of dopamine or noradrenaline though not quite as much as when prenylamine was added to the bath.

Table 1

The effect of prenylamine on the noradrenaline content of rabbit ileum (µg/g) and guinea-pig vas deferens (µg/horn) n = 4

Tissue	Stimulation with prenylamine	Stimulation without prenylamine
Rabbit ileum	0.322 ± 0.060	0.327 ± 0.057
Guinea pig vas deferens	0.809 ± 0.100	0.833 ± 0.103

As previously reported (HUKOVIC 1961) vasa deferentia from reserpine treated guinea pigs responded to stimulation of the hypogastric nerve, though the response decreased progressively with stimulation but was partially restored by the addition of dopamine or noradrenaline to the bath.

Analysis of segments of rabbit ileum or guinea pig vas deferens in which prenylamine in concentrations of 0.5 to 6 µg/ml had produced complete block of the response to sympathetic nerve stimulation showed no depletion of noradrenaline content (table 1)

Discussion

The results show that in vitro prenylamine blocks the response to the sympathetic nerves of rabbit intestine and guinea pig vas deferens and also antagonizes the response to added noradrenaline (rabbit intestine), this antagonism being reversible.

It is known that adrenergic receptor blocking agents antagonize added noradrenaline more readily than that released by the nerve on stimulation. Since blockade to sympathetic stimulation persisted when the response to added noradrenaline was restored (fig. 5c) failure of sympathetic stimulation to cause a response is unlikely to be due to the receptor blocking action of prenylamine. Further the receptor blocking action in segments of intestine taken from rabbits pretreated with prenylamine was very weak, while the response to sympathetic stimulation was still completely blocked. The indication that the failure of sympathetic stimulation to cause a response was due to impairment of the mechanism responsible for storing the transmitter is provided by experiments in which the addition of noradrenaline or dopamine restored the responses to almost control levels, though they gradually declined. This indicates that the

nerve terminals were still able to take up added amines but were unable to retain them

The ineffectiveness of prenylamine in vivo is difficult to understand, at least in the rabbit. There is no information on the effect of prenylamine on amine levels in the cat. It is possible that prenylamine does not readily deplete the storage granules in the cat

The capacity of prenylamine to deplete the amines in rat tissues is limited. After a single dose of 30 mg/kg (intraperitoneally) of prenylamine, the noradrenaline content of the heart is reduced to about 30 / of its normal value (OBIANWU 1965) Multiple doses (20 mg/kg twice daily for 3 days) only lowered the noradrenaline level of the heart by a further 8 / (unpublished result)

This limited capacity of prenylamine to deplete the amine storage granules in the rat (and possibly in the cat) may explain the total lack of effect on the sympathetic nerve function observed in the rat and cat.

The slight effect on sympathetic response in the rabbit is even more difficult to interprete. The dose of prenylamine used (8 mg/kg) has been reported to deplete the heart of its noradrenaline content by about 90 / (MACKENNA 1965) and further more, the sympathetic function was blocked in rabbits pretreated with a smaller dose of prenylamine (6 mg/kg intravenously) judged by the failure of stimulation of the periarterial nerves to elicit a response in the intestine. This fact lends support to a previous assumption (OBIANWU 1967) that anaesthetic agents used might have interfered with the action of prenylamine.

Another factor which may be of significance in explaining the slight (or lack of) action of prenylamine in vivo as compared with the in vitro effects is the fact that prenylamine potentiates some actions of nor adrenaline (OBIANWU 1967) Potentiation of the released transmitter may be of a magnitude sufficient to compensate for any depression of the release of the transmitter which prenylamine might have had

This will be particularly important in organs such as the nictitating membrane which has a rich adrenergic innvervation

The noradrenaline content of the rabbit nictitating membrane (0.176 μg/g, unpublished result) is not as high as that of the cat (4.29 μg/g, SMITH, TRENDELENBURG, LANGER & TSAI 1966) and this may indicate that the amount of the transmitter released per stimulation will be higher in the cat than in the rabbit and hence increased sensitivity to nor adrenaline will be of more pharmacological significance in the cat than in the rabbit

The observation that following prenylamine, injected noradrenaline and adrenaline caused a relaxation of the nictitating membrane of

the rabbit (fig 2) is very surprising. The nictitating membrane was supposed to be fully relaxed (since the cervical sympathetic trunk was cut) and any further relaxation is hard to imagine. This response was observed in 10 out of 12 experiments irrespective of whether prenylamine was given before or after anaesthesia. Involvement of either α or β-receptor seemed unlikely since phentolamine (5 mg/kg) and a new potent β-blocking agent (56/28) failed to influence the response

At present no explanation can be offered for this observation. Although the lens and the vitreous humor were expelled in each experiment in order to collapse the eye, a mechanical effect as a result of the movement of the eye cannot be ruled out

The rabbits were usually anaesthetized with urethane and pentobarbitone and it may be significant that BOWMAN GOLDBERG & RAPER (1962) reported that in the rabbit, urethane anaesthesia influenced the response of the soleus muscle to catecholamines Whether the response of the rabbit nictitating membrane to catecholamine after prenylamine can be attributed to the urethane anaesthesia is a matter for speculation.

In the intestine both α and β-receptors are present and involved in producing relaxation (or inhibition) of the gut (AHLQUIST & LEVY LEVY 1959) The relaxation of the intestine elicited by noradrenaline or adrenaline cannot be completely blocked either by α or β-receptor blocking agent alone, but can be completely blocked by a combination of the two agents (AHLQUIST & LEVY 1959) Prenylamine consistently abolished the responses of the rabbit intestine to adrenaline, noradrenaline and isoprenalinef(fig. 5) This block appeared to be reversible since it could be overcome by increasing the concentration of the sympathomimetic amines. Further evidence for the receptor blocking action of prenylamine is given in the next paper (OBIANWU 1967). Possibly due to potentiation of the actions of noradrenaline and adrenaline, the α receptor blocking action is not seen in vivo (see the paper referred to above for further discussion)

Failure of the sympathetic nerve to respond to stimulation in the isolated tissues could not be due to receptor block as this persisted when the receptor block had been overcome. In the rabbit nictitating membrane the mild depression of the sympathetic tone could not be a result of a receptor blocking action of prenylamine (since this is not of any significance in vivo) Any ganglion blocking action which prenylamine might have could not have influenced the result, since the responses of the nictitating membrane to pre or postganglionic stimulation after prenylamine were similar

The cause of failure of sympathetic nerve to respond to stimulation

might be an impairment of the mechanism responsible for the storage of amines in the granules at the nerve terminals. This view is supported by the previous observations (for references see OBIANWU 1965) that prenylamine, like reserpine impairs the storage capacity of the amine granules.

When block of the response to sympathetic nerve stimulation in the isolated tissues used was induced by prenylamine, there was no measurable loss of noradrenaline. This indicates that the onset of the block does not parallel the loss of measureable amounts of noradrenaline. A similar result was obtained with guanethidine in the same preparations by CASS & SPRIGGS (1961). ANDÉN, MAGNUSSON & WALDECK (1964) have demonstrated lack of correlation between sympathetic block induced by reserpine and amine levels.

These observations are consistent with the suggestion that only a small labile fraction of the amine pools is functionally essential (HÄGGENDAL & LINDQVIST 1963).

Summary

The adrenergic neurone and receptor blocking actions of prenylamine were investigated.

Sympathetic function was assessed by the contraction of the nictitating membrane to electrical stimulation of the cervical sympathetic trunk in the cat and rabbit, the retraction of the right inferior eyelid in the rat, contraction of the vas deferens in response to the stimulation of the hypogastric nerve and the relaxation of the rabbit intestine in the FINKLEMAN preparation.

In-vitro prenylamine in small concentrations completely abolished the response of the vas deferens and the rabbit intestine to sympathetic stimulation.

Vas deferens and intestine taken from animals pretreated with prenylamine (7–30 mg/kg) failed to respond to sympathetic stimulation.

Prenylamine only partially antagonized the contraction of the nictitating membrane of the rabbit and had no effect on that of the cat and the retraction of the inferior eyelid of the rat, in response to stimulation of the cervical sympathetic trunk.

The response of the nictitating membrane to added noradrenaline and adrenaline was potentiated in the cat and reversed in the rabbit.

Evidence for α- and β-receptor blocking action of prenylamine on rabbit isolated intestine was presented.

The discrepancy between the in vivo and in vitro effects of prenylamine is discussed.

The possible mechanism of action of prenylamine is discussed.

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Acta pharmacol et toxicol 1967 25 155–161

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(Professor Knud O Møller M D)

Interfering Substances in the Determination of Poisons in Autopsy Material 1 Hydroxymethyl-β-Carboline

By

Bent Kempe

(Received June 30, 1966)

In continuation of previous studies on substances produced during the process of Stas-Otto s extraction, we have now subjected to further analysis the substance long known by the name of animal chinoidin (JONES & DUPRÉ 1866 TRUHAUT & MOAN 1958), which in the preliminary analyses was found to behave like 1-hydroxymethyl-β-carboline (RR I no 2885) Its absorption curves in U V light have often been seen during analysis for alkaloids.

A description is given below of the isolation and identification of the compound, and methods are indicated for separating it from alkaloids, which are present in the same extraction phase.

Technique

Physico-chemical constants were determined as described previously (KEMPE 1964). The gas-chromatographic elementary analysis was carried out with an F & M Scientific CHN-analyzer, Model 185

A single hum liver stored for 25 days at 4–5° was used for analysis. The cause of death was barbituric acid poisoning (allypropymal (NFN) = aprobarbital (WHO)). 300 g minced liver tissue was extracted with ethanol, as described previously (KEMPE 1965). The hydrous tartaric acid extract btained was extracted with ether first with sulphuric acid ( ) and then at a strongly alkaline reaction (*b*) To the dehydrated ether phase obtained by the extraction at alkaline reaction, were added 2 drops of 8 N HCl. This was followed by evaporation t dryness on steam bath. The residue was heated with 30 ml of 0.1 N sulphuric acid on boiling water bath for bout 1 minute. The sulphuric acid solution was cooled to about 20° under the tap nd filtered. The filtrate was made alkaline with 2 N-NaOH and extracted three times, each time with an equal volume of chloroform. 8 N hydrochloric acid was added to the combined dehydrated chloroform extracts, after which

the chloroform was distilled off. The residue was purified by paper chromatography as described by BROBST *et al.* (1955) see table I

The development and dried chromatogram was observed in short-wave light (254 mμ). A blue-fluorescent streak was seen with an R_F value of 0.8. The blue-fluorescent area was cut out and subjected to descending elution with 0.2 N-HCl. The eluate (about 10 ml) was extracted twice at highly alkaline reaction, each time with 20 ml of chloroform. The chloroform was carefully distilled off on water bath, the last traces being removed by leaving the material in a porcelain dish at room temperature.

Results

An aliquot of the residue (about 1 mg) was dissolved in 0.1 N-HCl. The solution, which showed bluish fluorescence, was measured in a spectrophotometer at acid and alkaline reactions (see fig. 2) I R. see fig. 1

A compound with a similar type of absorption in the ultraviolet and infrared ranges is obtained by heating tryptophane in 1 N tartaric acid.

A) *Preparation of the compound (1-hydroxy-methyl β-carboline)*

To 100 mg L-tryptophane 40 ml of N tartaric acid and 10 ml of ethanol were added. The mixture was transferred to a porcelain dish, which was left on a boiling water bath for 10 hours. Evaporation to dryness was avoided by adding water The cooled solution was shaken three times at highly alkaline reaction (pH > 13) with an equal volume of chloroform The extract was purified by paper chromatography as described under isolation of the compound from liver tissue. The residue obtained weighed about 4 mg.

B) *Preliminary constitutional analysis of the synthesized compound*

The spectra of this compound were recorded, in ultraviolet light (as in fig. 2) and in infrared light (see fig. 1). Its micro-melting point after recrystallisation with methanol was estimated at 229° corr Its pK was calculated at 7 0 on the basis of spectrophotometric measurements of aqueous solutions of pH 5–8

An ordinary elementary micro-analysis gave 71 25 / C (72.74), 13 75 / N (14 14) and 5 00 / H (5 05) while a gas-chromatographic elementary analysis showed the following percentage values 72.4 C, 13 7 N and 5.2 H The bracketed figures represent the calculated percentages corresponding to $C_{12}H_{10}N_2O$ which is the formula of 1 hydroxymethyl-β-carboline.

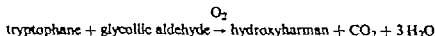
Since this compound is not listed in Chemical Abstract, we have subjected the synthesized substance to a preliminary constitutional analysis.

Fig. 1 Infrared spectra f the isolated substance (upper curve) and 1-hydroxymethyl-β-carboline (lower curve) in KB

The plotted absorption curves in ultraviolet light suggest that the compound contains a carboline nucleus (see fig. 2) The absorption band of the compound at 1035 cm $^{-1}$ (see fig. 1) probably indicates the presence of an alcohol group

Its nuclear magnetic resonance spectrum has been recorded. Comparison of this spectrum with that for 1 methyl-β-carboline (harman = aribine in the Merck Index, 7th edit. 1960, p 99) showed that the compound *contains an alcohol group, but neither a methyl group nor a formyl* group in position 1 Note that the alcohol band is not split in a triple band, as might be expected for a primary alcohol group

To obtain an additional evidence that the compound is 1-hydroxymethyl-β-carboline I produced it from glycollic aldehyde and tryptophane by a method commonly used for producing 1-substituted carboline derivatives (MANSKE & HOLMES 1952) The reaction formula is



$$\text{tryptophane} + \text{glycollic aldehyde} \xrightarrow{O_2} \text{hydroxyharman} + CO_2 + 3\,H_2O$$

the chloroform was distilled off The residue was purified by paper chromatography as described by BROWN *et al* (1955) see table 1

The development and dried chromatogram was observed in short-wave light (254 mµ). A blue-fluorescent streak was seen with an R_F value of 0.8. The blue-fluorescent area was cut out and subjected to descending elution with 0.2 N HCl. The eluate (about 10 ml) was extracted twice at highly alkaline reaction, each time with 20 ml of chloroform. The chloroform was carefully distilled off on a water bath, the last traces being removed by leaving the material in porcelain dish at room temperature.

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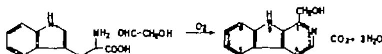


Fig. 3 Reaction between trypt phane and glycolaldehyde.

pound and that isolated from the liver are identical and presumably 1 hydroxymethyl-β-carboline

The distribution coefficients at room temperature for 1 hydroxymethyl β-carboline in 0 1 N NaOH/ether and 0 1 N NaOH/chloroform are 0 1 Since it is extracted like alkaloids and shows a spectrum that distorts the spectra of the alkaloids, its R_F value and its reaction with commonly used alkaloid reagents, have been tested.

C) R_F value of 1-hydroxymethyl-β-carboline and its reaction with alkaloid reagents

The R_F values and colour reactions of 1 hydroxymethyl-β-carboline and some commonly occurring alkaloids are shown in table 1

Table 1

R_F values and colour reactions of 1 hydroxymethyl-β-carboline and some commonly occurring alkaloids on Whatman's paper no. 1 prepared with 0 1 M phosphate buffer f pH 6.3 (B)

Ascending development.
Developing liquids
System A n-Butanol, glacial acetic acid and water 40 10 50 (PARTRIDGE & WESTALL 1948) The chromatogram saturated for 45 minutes (lower phase) and developed for 17 hours (upper phase)
System B Amylene hydrate, di-n-butylether and water 80 7 13 (BROSS et al 1955).
Reagents 1 Iodoplatinate
2. Dragendorff reagent

Substance	R_F in developing liquid A	R_F in developing liquid B	U V light	Reagent 1	Reagent 2
1-hydroxymethyl-β-carboline	0.73	0 78	bluefluoresc.	dark	orange
Quinine	0.83	0.47	bluefluoresc	bluish-violet	orange
Nicotine	0.50	0 45	bsorb.	bluish-violet	orange
Codeine.	0.56	0 13	absorb	bl ish-violet	orange
Morphine	0.50	0 06	bsorb.	blue	orange
Pethidine	0 82	0.58	0	bluish-violet	orange
Methadone	0.92	0.45	0	violet	ora ge
Strychnine	0.66	0.22	absorb	bluish-violet	orange

Acta pharmacol et toxicol 1967 25, 162–168

From the Department of Pharmacology University of Copenhagen
(Professor Knud O Møller M D.)

Studies on the Subcutaneous Absorption in Mice
I A Method for Studying Quantitatively the Dynamics of Subcutaneous Absorption*

By

E. Secher-Hansen, H. Langgård and Jens Schou

(Received September 29 1966)

A method is described for the quantitative investigation of the absorption of water and water-soluble substances from a subcutaneous "depot"

The method was especially designed to follow simultaneously the decrease in the surplus of water introduced into the tissues by injections, and the exchange of the injected water molecules with plasma water

Method

Male albino mice of a single strain (Leo, Stritt), 4–6 weeks old were used. While the animals were under light halothane (fluothane ®) anaesthesia, two semilunar symmetrical 5.5 cm² areas were marked out on the depilated skin f the back (fig. 1 cf. further SZPORNY LANGGÅRD & HVIDBERG 1964). Using a micrometer syringe (Agla ®) a standard olume of 80 μl of 0.9% N Cl, 10% sucrose or 14% sucrose in water containing about 3 μc of tritiated water ($^{3}H_2O$ New England Nuclear Corp.) was injected subcutaneously within the limits of the right-side rea (fig 2) After 5 or 15 minutes the animal was stunned by a blow on the neck, decapitated and bled. The two pieces of skin marked, including the subcutaneous tissue, were cut out and weighed immediately on a torsion balance. The part of the volume njected, which remained at the injection site at the end of the experimental period (the residual volume) was determined as the difference i weight between the two sides and expressed as per cent of the volume injected (the residual volume per cent). For the measurements of radioactivity each skin sample was hydrolyzed ith a number of μl of 0.5 N-NaOH corresponding to 3 times the weight in mg of the skin sample. The proteins were precipitated by additio of an equal olume of 10% ZnSO After centrif gation 100 μl of the clea supernatant was dded to 4 ml of scintillation medium

*) A preliminary report f this nvestigation was given at the Third International Pharmacological Congress, São Paulo Brazil, July 1966 Abstract vol. p. 153 No. 387



Fig. 1 Halothane anaesthetized mouse, marked with two symmetrical, 5.5 cm² areas on the depilated skin of the back.

(BRAY 1960). The radioactivity was measured in a liquid scintillation counter (Isotope Developments Ltd.)

The radioactivity remaining at the injection site (the residual radioactivity) was determined as the difference between the total radioactivity of the injected and the uninjected skin samples. Thus, correction was made for the background counting and for radioactivity due to recirculation. The residual radioactivity was expressed as per cent (residual radio-



Fig. 2. Injection into the subcutaneous tissue.

activity per cent) of the injected dose, measured as the radioactivity of an excised skin sample injected with the standard dose, and corrected for background counting.

To examine the error of the cutting technique, weight and composition of the excised right and left side skin samples from a group of uninjected mice were examined.

To determine whether the injections were given quantitatively within the limits of the excised skin sample the same measurements as stated above were made in a group of mice injected with the standard volume (80 µl 0 9 % N Cl) immediately *after* the animals had been killed and bled.

Results

The figures in table 1 show the results of the first control experiment. It is seen that from uninjected mice two marked skin areas can be cut out with identical weight and composition

Table 1

Weight and composition of two symmetrical pieces of skin from uninjected mice (control group 1 number of animals: 12)

	Total Weight mg Mean	Weight Diff % Mean s.e.m.	Water mg Mean s.e.m.	Fat mg Mean s.e.m.	Fat free Solid mg Mean s.e.m.
Right side	265	0.6 ± 1.3	166 ± 3 9	39 ± 5.2	60 ± 1.6
Left side	263		162 ± 3.1	41 ± 4.9	60 ± 1.2

The figures in table 2 show the difference between the two sides when 80 µl 0 9 / NaCl had been injected subcutaneously into the right side of dead animals. The difference in weight was on an average 80 mg and the content of water was on an average, 80 mg higher on the right than on the left side (The injected NaCl weighed 0.7 mg.)

Table 2

Weight and compositio of two symmetrical pieces of skin from killed mice in which 80 µl 0 9 saline was injected subcutaneously on the right side (*control group 2, number of animals 8*)

	Total Weight mg Mea	Weight Diff. Total mg	Water mg Mean s.e.m.	Weight Diff Water mg Mean s.e.m.	Fat mg Mean s.e.m.	Fat free Solids mg Mean s.e.m.
Right Side	319	80	233 4 9	80 ± 2.3	27 5.3	59 ± 1.2
Left Side	239		153 4 8		28 5 6	58 ± 1.6

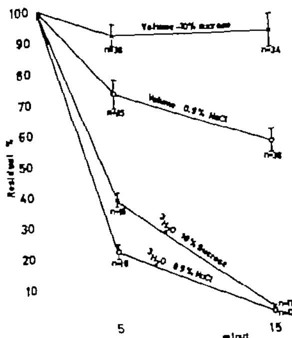


Fig. 3 Ordinate Residual volume (upper two curves) or residual radioactivity (lower tw curves) of 80 µl of 10% sucrose and 0.9% NaCl in water containing pproximately 3 µc of ^3H_2O, 5 and 15 minutes (abscissa) after subcutaneous injections into the right area of two symmetrical areas of depilated skin on the back of mice. The residual values are determined as the difference in weight (volume) and radioactivity between the injected and the uninjected sides, and are expressed in per cent of the total dose injected. The standard errors of the means are indicated by vertical lines.

Figure 3 illustrates graphically the percentual reduction in volume and radioactivity 5 and 15 minutes after the injections of 80 µl of either 0.9 / NaCl or 10 / sucrose solutions, containing approximately 3 µc of ^3H_2O It is seen that the decrease in volume was faster for the sodium chloride solution than for the sucrose solution. Furthermore the water molecules were exchanged more rapidly in the 0 9 / NaCl than in the 10 / sucrose environment In both cases, however the water molecules were exchanged rapidly while the reduction of the volume injected was a much slower process. After 15 minutes, nearly all the originally injected water molecules had disappeared, while the volume injected still remained after the sucrose injections and more than half the volume persisted after the injection of sodium chloride.

Figure 4 shows the same values five minutes after injection of 10 or 14 sucrose. The lowest residual radioactivity was found in the experiment with the highest residual volume, i.e. with the hypertonic solution.

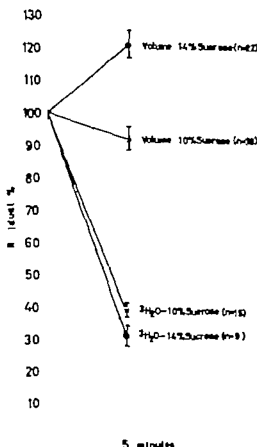


Fig. 4. Ordinate Residual volume (upper curves) or residual radioactivity (lower curves) of 80 µl of 14 % or 10 sucrose I water containing approximately 3 µc of ^3H_2O, 5 minutes (bscissa) after subcutaneous injections into the right f two symmetrical areas of depilated skin on the back f mice. The residual values are determined as the difference in eight (volume) and radioactivity between the injected and the uninjected sides, and are expressed in per cent of the total dose injected. Th standard errors of the means are indicated by vertical lines.

Discussion

Examination of the kinetics of subcutaneous absorption may be based on different principles. One of the earliest of these was the papule-disappearance method (MC CLURE & ALDRICH 1923) by which BARRON *et al* (1951) demonstrated that there is no good agreement between the reduction of physiological ^{24}NaCl injected intracutaneously and the reduction in volume of the papule produced. The method is, however more relevant to the absorption from the corium.

A summary of methods and principles formerly used for estimating

the rate of the subcutaneous absorption has been given by SCHOU (1961) Most of the methods are only semi-quantitative. During recent years extensive use has been made of γ radiating tracer substances by which quantitative data on the local absorption rate can be obtained. Such investigations offer the advantage that the radioactivity can be measured continuously and from the surface, but are inadequate with regard to geometrical conditions and spread. The correct determination requires excision of the injection zone at the time of observation and chemical or radio-chemical analyses of the remaining amount of the substances injected.

The main advantage of the present method depends, however on the marking and cutting in the same animal of two symmetrical pieces of skin, of which one is injected, and the other uninjected. The results of the control experiments (table 1 and 2) show that with the method described, it is correct to use the uninjected side as a control of the injected side. It is possible to measure simultaneously the decrease in the amount of a test substance and the decrease in the volume injected. Furthermore, the rate at which the injected water molecules (electrolytes or any other component) is actually removed from the injected zone by exchange between the "depot" and the circulating plasma of the blood can be determined. The method is therefore well suited for studying the kinetic relations in the loose interstitial connective tissue during subcutaneous absorption and has been particularly developed for examining the significance of the ground substance in such respect.

The volume of 80 µl was chosen as a compromise. On the one hand, it was hoped to inject as large a volume as possible so that the weighing of the residual volume could be made with sufficient accuracy on the other hand, to have a volume so small that the continuity of the tissue and its functional capacity would be injured as little as possible Experiments with injections of dye-substances and radiochemical analysis of adjacent areas (unpublished results) showed that the volume injected was localized exclusively in the subcutaneous tissue and that during the test period this did not spread beyond the area marked.

The fact that a volume of 0 9 / NaCl reduces faster than a corresponding volume of blood-isotonic (10 %) sucrose solution (fig. 3) is presumably due to the slower movement of the sucrose molecules in the ground substance. The more rapid exchange of water molecules in the sodium chloride solution than in the sucrose solution, might be linked to the faster volume disappearance rate of the sodium chloride "depot" including a solvent drag (filtration) However fig. 4 shows that water molecules are exchanged more rapidly from a hypertonic sucrose solution than from the blood-isotonic solution, in spite of the fact that the former volume increases significantly during the observation period. The in-

creased capillary permeability and thus the fraction of capillary surface accessible to water diffusion due to the injection of a bloodhypertonic solution too cannot explain the finding (unpublished data). A possible explanation may be that some of the water molecules in the sucrose solution are attached to the sucrose molecules as water of crystallization so that they will move along with these at the slower rate.

The experiments demonstrated in fig. 3 and 4 show distinctly that the exchange of water molecules and the disappearance of the volume injected are not parallel processes. Various stimuli may even influence them in opposite directions. Experiments are in progress, using the reported method for investigating how various systemic and local factors are influencing the two processes.

Summary

A method has been developed by which the reduction of a subcutaneously injected surplus of water can be followed simultaneously with the rate of exchange of the water molecules between the depot and the plasma water

Using this method it is shown that the disappearance of the volume injected and the exchange of the water molecules are independent processes and that various stimuli such as a change in tonicity of the injected fluid, may influence them in opposite directions.

Acknowledgements

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Interference of Intra-arterially Administered Phenoxybenzamine, Guanethidine and Hydralazine with the Adrenergic Vasoconstrictor Function in the Hand
A Comparative Study in Man

By

Matts Hentling and Gillis Johnsson

(Received January 11 1967)

The vasodilator effects of phenoxybenzamine and guanethidine are considered to be due to peripheral sympathetic blockade. Numerous animal studies indicate that phenoxybenzamine acts through α-adrenergic receptor blockade (NICKERSON *et al* 1951 review by NICKERSON 1959) while guanethidine exerts its action by peripheral adrenergic neuron blockade (MAXWELL *et al* 1960 MCCUBBIN *et al* 1961) Studies in man of the regional vascular effects of intra-arterially administered phenoxybenzamine (DUFF & GINSBURG 1957 GINSBURG & DUFF 1958 DE LA LANDE & WHELAN 1959 ALLWOOD & GINSBURG 1961 LOWE & ROBINSON 1964) and guanethidine (COOPER *et al.* 1963) indicate that the two drugs elicit vasodilatation through the same mechanisms as have been demonstrated in animal studies. However the doses used in the human pharmacological studies were relatively large and the local tissue concentration of the drug might have exceeded the concentration attained after systemic administration of the drugs in therapeutic doses.

In a previous communication from this laboratory (ÅBLAD *et al* 1962) a description was given of the mechanism by which hydralazine administered into a brachial artery in different doses, interfered with the adrenergic vasoconstrictor function in the hand. It was found that hydralazine, given in low doses, elicited significant vasodilatation without markedly antagonizing the adrenergic vasoconstrictor responses, but when larger doses were given these responses were to a great extent antagonized

In the present investigation a similar study has been performed using small doses of phenoxybenzamine and guanethidine, and the results have been compared with those obtained with hydralazine. The vasodilator drugs have been administered intra-arterially into a brachial artery and their interference with the vasoconstrictor effects of intra-arterially and intravenously infused noradrenaline and a cold test have been compared. The findings in this study have been briefly mentioned in a previous report (ÅBLAD 1963)

Methods

The study was performed on fourteen healthy recumbent students at room temperature of 27 ± 1°C. The blood flow in the hands was measured with the venous occlusion plethysmograph as previously described (ÅBLAD *et al* 1961 & 1962). Thin polyethylene catheters were inserted into one brachial artery and one cubital vein (ÅBLAD *et al.* 1961). The catheters were connected to motor-driven syringes delivering isotonic saline or test drugs dissolved in isotonic saline at a constant rate (0.6 ml/minute). Each experiment proceeded according to the following pattern. Blood flow was determined simultaneously in both hands once or twice a minute. When the basal blood flow had reached a stable level, control responses were recorded to a cold test and to noradrenaline infused intravenously or intra-arterially for five minutes. The cold test consisted of placing a piece of ice in the subject's mouth for 70 sec. The intervals between the vasoconstrictor stimuli were always more than 10 minutes, and care was taken to ensure that the effect of one stimulus had disappeared before the next one was given. The order between the infusions of noradrenaline and the cold test was alternated in different experiments. Once control responses to the vasoconstrictor stimuli had been obtained, phenoxybenzamine or guanethidine was infused intra-arterially for five minutes. When the effect of these drugs on hand blood flow was constant, the vasoconstrictor tests were repeated. In three of the subjects the same test procedure was repeated at a later date, but instead of blood flow arterial blood pressure was measured using a conventional auscultatory technique.

The drugs used were l-noradrenaline bitartrate in a dose of 14–20 µg per minute (in one experiment 10 µg per minute) given intravenously or 0.28–0.30 µg per minute (in one experiment 0.09 µg per minute) given intra-arterially Guanethidine bisulfate (Ismelin ®, Ciba) was administered in a total mean dose of 1 3 mg (range 0.5–2.0 mg). Phenoxybenzamine hydrochloride (dibenzyline ®, Smith, Kline and French) was given in a total mean dose of 0.04 mg (range 0.02–0.10 mg). The doses of the intra-arterially infused vasodilator drugs were so adjusted that the blood flow increase in the ipsilateral hand would be about the same after phenoxybenzamine and guanethidine as after a low dose (0.30–0.55 mg) of hydralazine in a corresponding study (ÅBLAD *et al* 1962).

The individual "basal" values for blood flow in the results are the mean of 4–5 blood flow determinations made over the two-minute period immediately before the application of a vasoconstrictor stimulus. The values representing the blood flow during infusions of noradrenaline are the mean of, as a rule, 5 determinations made during the last two minutes of the infusion. The cold test blood flow was determined 10, 40 and 60 seconds after application of the cold stimulus.

According to WILKINS & EICHNA (1941) the initial effect of the cold test on the peripheral blood flow is due solely to increased activity in the vasoconstrictor fibres, but after about 30 seconds the peripheral circulation is sometimes further influenced by some humoral

factor released from the adrenals, probably adrenaline. Therefore in our results the effect of the cold test on blood flow in the hand is given both as the value observed 10 seconds after the application of the stimulus and as the mean of those recorded after 40 and 60 seconds.

Results are reported as means ± standard error of the mean. The statistical analysis was based on the t test (FISHER 1958) for differences in subjects. The analyses were as a rule performed on the differences between the absolute blood flow changes in the two hands induced by the different stimuli. The results obtained in the studies with phenoxybenzamine and guanethidine were compared with those reported previously for hydralazine (ÅBLAD *et al.* 1962).

Results

A. *Effects of intra-arterially administered phenoxybenzamine and guanethidine on blood flow*

Seven experiments were performed with *phenoxybenzamine*. After the administration of the drug into one brachial artery the blood flow in the same hand (test hand) began to rise within a few minutes after starting the infusion. The increase in the test hand blood flow reached its maximum in 30–60 minutes and the vasodilator effect then remained essentially unchanged in all subjects throughout the remainder of the experiment (about two hours). The blood flow in the other hand (control hand) was not significantly altered. No evidence of systemic effects of the drug was obtained. The results are given in table 1. Calculation of the influence of phenoxybenzamine on the blood flow recorded before the application of vasoconstrictor stimuli provided an estimate of the vasodilator effect of the drug.

Table 1

Effects of intra-arterially infused phenoxybenzamine and guanethidine on the blood flow in the hands. The drugs were infused into the brachial artery of the test hand side. Blood flow in ml per 100 ml tissue per minute.

	Before vasodilating drug			After vasodilating drug		
	Test hand	Control hand	Difference test-control	Test hand	Control hand	Difference test-control
Phenoxybenzamine n = 7	19.6 ± 2.16	18.7 ± 2.24	0.9 ± 0.68	27.1 ± 2.21	20.6 ± 1.75	6.5 ± 1.15 $P < 0.01$
Guanethidine n = 8	18.4 ± 1.87	18.3 ± 1.98	0.1 ± 0.58	4.7 ± 1.26	18.7 ± 2.2	6.0 ± 2.04 $P < 0.05$

Eight experiments were performed with *guanethidine* In one of these (dose of guanethidine 2.0 mg) we noted an initial decrease in the test hand blood flow beginning 2 minutes after the start of infusion and lasting for some 15 minutes. This was followed by an increase above the initial level. In the other seven experiments an increase in blood flow was seen a few minutes after the start of the infusion The test hand attained a peak blood flow after about 30 minutes and then always remained practically unchanged throughout the remainder of the experiment (about two hours). There was no evidence of any significant changes in the blood flow in the control hand or any systemic effects of the drug. The results are presented in table 1 The calculation of the vasodilator effect of the drug is performed as for phenoxybenzamine, as mentioned above.

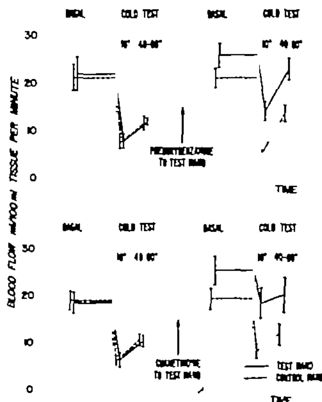


Fig. 1 Effect of cold test on hand blood flow before and after administration of phenoxybenzamine (mean of 5 experiments) and guanethidine (mean of 7 experiments) into the brachial artery of the test hand side (solid line). The blood flow of the other hand served as control (dashed line). Mean ± s.e.m.

Table 2

Decrease of blood flow in the hands produced by vasoconstrictor stimuli before and after infusion of phenoxybenzamine intra-arterially on the test hand side. Blood flow *decrease* 1 ml per 100 ml tissue per minute.

Vasoconstrictor stimulus	Before phenoxybenzamine			After phenoxybenzamine		
	Test hand	Control hand	Difference test-control	Test hand	Control hand	Difference test-control
Cold test, 10 sec. = 5	14.0 ± 2.61	13.9 ± 2.37	+0.1 ± 0.72	11 7 ± 2.40	16.1 ± 2.41	−4 4 ± 1.40 P < 0.05
Cold test, 40–60 sec. = 5	10.1 ± 3.29	9.5 ± 2.82	+0.6 ± 1.38	2.6 ± 2.65	7 6 ± 2.65	−5.0 ± 0.89 P < 0.01
Noradrenaline intravenously = 5	5.9 ± 1.80	6.6 ± 2.26	−0.7 ± 0 66	2.1 ± 2 02	10.4 ± 2.06	−8.3 ± 1.59 P < 0 01

B *Effects of phenoxybenzamine and guanethidine on the vasoconstrictor action of the cold test in the hand*

The cold test had little effect on the blood pressure. In the three experiments in which this factor was studied the systolic and diastolic blood pressure increased by 0–10 mm Hg.

1) Phenoxybenzamine

In five experiments the effect of the cold test on blood flow was investigated before and after infusion of phenoxybenzamine (Fig. 1 Table 2) Before the infusion of the drug, the cold test produced a quantitatively similar reduction in blood flow in the two hands after 10, 40 and 60 seconds. After the intra arterial administration of phenoxybenzamine the cold test produced a significantly smaller decrease in blood flow in the test hand than in the control hand

2) Guanethidine

The effect of guanethidine on the vasoconstrictor action of the cold test was studied in seven experiments (Fig. 1 Table 3) Before the infusion of guanethidine the cold test produced a quantitatively similar reduction of

Table 3

Decrease of blood flow in the hands produced by vasoconstrictor stimuli before and after *infusion* of guanethidine intra-arterially on the test hand side. Blood flow *decrease* in ml per 100 ml tissue per minute.

Vasoconstrictor stimulus	Before guanethidine			After guanethidine		
	Test hand	Control hand	Difference test-control	Test hand	Control hand	Difference test-control
Cold test, 10 sec. n = 7	12.1 ± 1.99	12.8 ± 1.71	−0.7 ± 0.32	6.9 ± 1.53	11.2 ± 1.63	−4.3 ± 1.02 P < 0.01
Cold test, 40–60 sec. n = 7	8.0 ± 1.35	8.3 ± 1.06	−0.3 ± 0.54	5.3 ± 1.88	7.6 ± 2.00	−2.3 ± 1.75
Noradrenaline intravenously n = 7	7.0 ± 1.90	6.6 ± 2.22	+0.4 ± 0.63	9.4 ± 1.57	7.3 ± 1.75	+2.1 ± 0.57 P < 0.02

blood flow in the two hands. When the cold test was applied *after* the intra-arterial administration of guanethidine, it produced a significantly smaller decrease of blood flow in the test hand than in the control hand after 10 seconds. The cold test responses after 40–60 seconds were, however, not significantly different in the two hands.

C. *Effects of phenoxybenzamine and guanethidine on the vasoconstrictor action of noradrenaline in the hand*

An intravenous infusion of 14–20 μg of noradrenaline bitartrate raised the systolic blood pressure by 10–25 mm Hg and the diastolic by 15–35 mm Hg. In the doses used, intra-arterially administered phenoxybenzamine or guanethidine did not change the blood pressure response to intravenous noradrenaline. Intra-arterially infused noradrenaline had no effect on the arterial blood pressure or on the control hand blood flow.

1) Phenoxybenzamine

In five experiments the effect of an intravenous infusion of noradrenaline on the blood flow in the hand was studied before and after intra-arterial

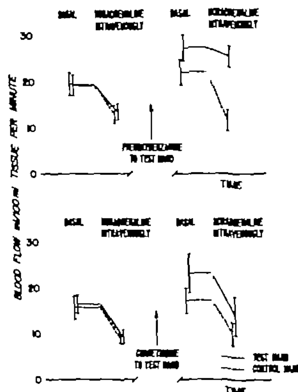


Fig. 2. Effect of intravenous infusion of noradrenaline hand blood flow before and after administration of phenoxybenzamine (mean of 5 experiments) and guanethidine (mean of 7 experiments) into the brachial artery of the test hand side (solid line). The blood flow of the other hand served as control (dashed line). Mean ± s.e.m.

administration of phenoxybenzamine (Fig. 2, Table 2) Noradrenaline decreased the blood flow in the two hands to about the same extent as before the administration of phenoxybenzamine. After this drug, nor adrenaline produced a significantly smaller decrease of the blood flow in the test than in the control hand.

Three subjects received both noradrenaline and phenoxybenzamine intra-arterially into the test hand (Fig. 3). Phenoxybenzamine considerably reduced the vasoconstrictor response to noradrenaline in the test hand in all subjects. The noradrenaline-induced decrease of test hand blood flow after phenoxybenzamine was 4.2 ± 0.41 ml/100 ml tissue/minute (in the following abbreviated as ml) as compared to 10 0 ± 1.50 ml before the administration of phenoxybenzamine.

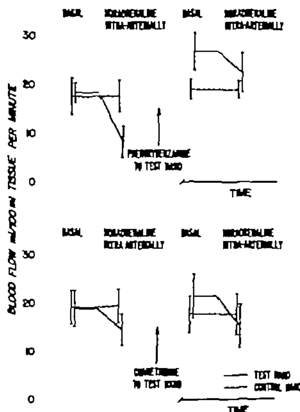


Fig. 3 Effect of intra-arterial infusion of noradrenaline into the brachial artery of the test hand side (solid line) on blood flow in the hand before and after administration of phenoxybenzamine (mean of 3 experiments) and guanethidine (mean of 4 experiments) into the same artery The blood flow of the other hand served as control (dashed line). Mean ± s.e.m.

2) Guanethidine

In seven experiments the effect of an intravenous infusion of noradrenaline was studied before and after intra-arterial administration of guanethidine (Fig. 2, Table 3) Before guanethidine noradrenaline produced essentially the same reduction in blood flow in the two hands. After guanethidine, however noradrenaline produced a greater decrease in flow in the test than in the control hand. In the control hand the effect of noradrenaline was about the same as before guanethidine

In four experiments, both noradrenaline and guanethidine were given intra-arterially into the test hand (Fig. 3) After guanethidine the blood flow decrease elicited by noradrenaline in the test hand tended to be more marked than that seen before the vasodilator agent was given. The noradrenaline-induced decrease of test hand blood flow after guanethidine was 6.1 ± 1 04 ml compared with 4.2 ± 1.93 ml before guanethidine.

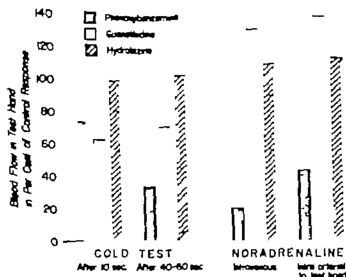


Fig. 4 Effect of intra-arterially administered phenoxybenzamine (0.02–0.10 mg), guanethidine (0 50–2.00 mg) and hydralazine (0.30–0.55 mg) on the vasoconstrictor responses to a cold test and t noradrenaline.

The effect of the vasoconstrictor stimuli in the hand treated with a vasodilator drug (test hand) is expressed in per cent of the control blood flow response as follows. For the cold test and intravenously infused noradrenaline the vasoconstrictor response in the test hand was related to the simultaneously occurring vasoconstrictor response in the untreated contralateral hand (control hand). For intra-arterially infused noradrenaline the vasoconstrictor response in the test hand was related to the response produced by the same dose of noradrenaline in the test hand before it was treated with vasodilator drug.

D *Comparison between phenoxybenzamine guanethidine and hydralazine*

It was of interest to compare the above results with those obtained in an identical study with hydralazine (intra-arterial dose 0.30–0 55 mg, ÅBLAD *et al* 1962) In the doses given all three drugs produced about the same increase in blood flow in the test hand while the blood flow in the control hand was not significantly changed. This probably implies that the three drugs produced about the same mean decrease of the vascular resistance in the test hand, since the average basal hand flow recorded before the drugs were given was similar in the three series. The average control responses to the respective vasoconstrictor stimuli were also of the same order of magnitude in the three series. Fig. 4 summarizes the manner in which the three vasodilator drugs influenced the response to the vasoconstrictor

stimuli in the hand. The interference of the vasodilator drugs with the responses to the cold test and to intravenously administered noradrenaline was calculated by relating the induced blood flow decrease in the vasodilator treated test hand to the simultaneously recorded decrease in the control hand on a percentage basis. The influence on the response to intra arterial noradrenaline is represented by the percentage relationship between the noradrenaline induced reductions of test hand blood flow after and before vasodilator treatment. Consideration is taken of spontaneous changes in the hand blood flow as recorded in the control hand.

On the basis of these data it should be possible to make an approximate comparison between the three vasodilator drugs with regard to their effect on the adrenergic control of vascular resistance in the hand Phenoxybenzamine and guanethidine inhibited the "10th second" cold test response to about the same extent, but phenoxybenzamine seemed to have a more marked effect than guanethidine on the "40th–60th second" cold test response. Phenoxybenzamine tended to inhibit the noradrenaline-induced effect more than the 10th second" cold test response. Guanethidine on the other hand elicited, if anything, a slight increase of the noradrenaline effect on the blood flow Hydralazine differed from both phenoxybenzamine and guanethidine in that it produced vasodilatation without significantly influencing the response to either the cold test or the noradrenaline infusion.

Discussion

The results show that intra arterially administered phenoxybenzamine and guanethidine produced a relatively long-lasting decrease of vascular resistance in the hand. The drugs were administered in low doses so that their concentration in the hand tissues could be assumed to be about the same, or even less in the case of phenoxybenzamine than the concentrations attained after systemic administration of the drugs in therapeutic doses (cf MAXWELL 1965) Thus the results are in agreement with the accepted view that the vasodilator effect of both phenoxybenzamine and guanethidine is mainly due to an action on the peripheral vascular bed. The vasodilator effect of hydralazine has also been shown to be mainly due to an action on a peripheral site (STUNKARD *et al* 1954 ÅBLAD 1959 ÅBLAD *et al* 1961 ÅBLAD 1963). However the three drugs appeared to act through different mechanisms since their interaction with adrenergic vasoconstrictor stimuli resulted in the production of different patterns of response.

The vasodilator effect of phenoxybenzamine was accompanied by reduced responses to both the cold test and infused noradrenaline. This result is compatible with the view that the vasodilator action of phenoxybenzamine was mainly due to blockade of the adrenergic α receptors. Phenoxybenzamine appeared to antagonize the cold test response after 10 seconds to a smaller extent than the response to noradrenaline. This finding suggests that the drug gave more marked inhibition of the effect due to circulating noradrenaline, than of that due to adrenergic nerve stimulation, as previously found in animal experiments (YOUMANS *et al.* 1955, NICKERSON 1959).

The vasodilator effect of guanethidine was accompanied by a significantly reduced response to the cold test after 10 seconds, whereas the blood flow decrease due to infused noradrenaline was, if anything, accentuated. The blockade by guanethidine seemed to be more marked 10 seconds after the application of the cold test than after 40 and 60 seconds. Circulating catecholamines may have contributed to the vasoconstriction seen at the later period (WILKINS & EICHNA 1941). The results are in agreement with the view that the vasodilator effect of guanethidine is due to a decreased release of noradrenaline from the vasoconstrictor nerve endings, *i.e.* adrenergic neurone blockade.

Hydralazine differed from phenoxybenzamine and guanethidine in that it elicited the same vasodilatation without markedly antagonizing any of the vasoconstrictor stimuli. This finding supports other data indicating that the vasodilator effect of hydralazine is primarily due to a direct action on the autonomous "myogenic" vascular tone (review by ÅBLAD 1963).

By using *low doses* of phenoxybenzamine, guanethidine and hydralazine it was thus possible to obtain a clearcut differentiation between their respective mechanisms of action and to demonstrate the main factor involved in their vascular effects. Administration of larger intra-arterial doses could be expected to make such a characterization more difficult. Phenoxybenzamine, given intra-arterially in amounts higher than those used in the present study, caused a vasodilatation in the sympathetic denervated hand which was ascribed to a peripheral action independent of any effect the drug might have on the sympathetic nervous system (DUFF 1956). It is well known from *in vitro* studies that phenoxybenzamine is an α-adrenergic receptor antagonist with limited specificity (FURCHGOTT 1955), since it also acts as a potent histamine and serotonin antagonist.

In the present study the vasodilator effect of guanethidine, in a dose of 0.5–2.0 mg intra-arterially, was evident within a few minutes of the start of the infusion. However, in one experiment, in which the dose of guanethidine was 2.0 mg, there was an initial vasoconstriction which lasted for

about 15 minutes. COOPER *et al* (1963) found that intra-arterial doses of 5 mg of guanethidine produced a long-lasting vasoconstriction in the hand. These investigators also found that guanethidine (in the higher dose) accentuated the effect of noradrenaline on the hand blood flow more than we observed in the present study These studies confirm other studies indicating that guanethidine has complex actions on the adrenergic nerve terminal The mechanisms of these various actions of guanethidine are not completely clear Guanethidine may have a reserpine-like effect on the transmitter store (CASS *et al* 1960) but the adrenergic blockade seems to start long before depletion is complete (MUSCHOLL 1965) This apparent discrepancy may be resolved if we assume that guanethidine primarily causes a depletion of the small labile, functionally essential fraction of the transmitter store (cf CARLSSON 1966b). Guanethidine has also been found to elicit a blockade of the catecholamine neuronal re-uptake (for detailed discussion and references see MALMFORS 1965 CARLSSON & WALDECK 1965 CARLSSON 1966a & b) This blocking effect of guanethidine on the "membrane pump" may explain the augmented effect of guanethidine on the noradrenaline response and together with the reserpine like effect of the drug, this action may explain the initial vasoconstrictor response of guanethidine (cf CARLSSON 1966b) It has further been suggested that higher doses of guanethidine produce vasodilatation also through β-adrenergic receptor activation (ABBOUD & ECKSTEIN 1962) and by means of unspecific depression of vascular "myogenic" tone (ABBOUD *et al* 1961)

In a previous study (ÅBLAD *et al* 1962) it was found that larger amounts of hydralazine (0 95–1 27 mg intra arterially) than those referred to in the comparison made above, not only produced a more marked vasodilatation in the hand but also evoked a pronounced inhibition of the vasoconstriction caused both by the cold test and by noradrenaline infusion (ÅBLAD *et al* 1962) This finding indicates that the vasodilator effect of the higher doses of hydralazine is in part due to inhibition of adrenergic vasoconstrictor tone. This inhibition may represent a pronounced depression of vascular "myogenic" tone and can by no means be interpreted as indicating that hydralazine is an α-adrenergic receptor antagonist.

Summary

Infusions of phenoxybenzamine (0 02–0 10 mg), guanethidine (0.5–2.0 mg) and hydralazine (0 30–0.55 mg) into the brachial artery of normal subjects elicited an increase of hand blood flow characterized by slow onset and long duration. In the doses used, all three drugs produced an increase in hand blood flow (about 30–50 ⁄) but they differed in their

responses to the vasoconstrictor effects of a cold test and to intravenously or intra-arterially infused noradrenaline. Phenoxybenzamine inhibited the response to both the cold test and to noradrenaline infusion Guanethidine weakened the effect of the cold test whereas the noradrenaline response was, if anything, augmented. Hydralazine did not significantly influence the effects of the vasoconstrictor stimuli.

The results demonstrate three different ways of reducing vascular resistance. Phenoxybenzamine probably acted by α-adrenergic receptor blockade, guanethidine by adrenergic neurone blockade and hydralazine by a "direct action on the vascular "myogenic" tone.

Other effects of the vasodilator drugs, particularly after administration of larger doses, are discussed. The doses of the three vasodilator agents used in the present study might be considered as giving a drug concentration in the hand in the same range as that obtained after systemic administration of the drugs in clinically used doses. The authors consider this important for a relevant analysis of the mechanisms of the therapeutic action of a vasodilator drug.

Acknowledgements

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The In Vitro Inactivation of Histamine in Kidney Tissue of Sheep

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(Received September 19 1966)

In intact animals histamine is mainly inactivated by two major pathways.

1) Primary oxidative deamination leads to the formation of imidazole 4-acetic acid (MEHLER, TABOR & BAUER 1952 TABOR, MEHLER & SCHAYER 1953). The initial step in this reaction is carried out by histaminase (diamine oxidase) Imidazole-4-acetic acid is partly conjugated with ribose before being excreted in the urine (TABOR & HAYAISHI 1955)

2) Methylation of the ring nitrogen remote from the sidechain, is partly followed by secondary oxidative deamination of the side-chain (SCHAYER & KARJALA 1956)

In all species examined inactivation by both pathways has been demonstrated (SCHAYER, KENNEDY & SMILEY 1953 SCHAYER 1959) though in the rat, only a small fraction of the substance is methylated (SCHAYER 1952 WESTLING 1958).

Of the species previously examined, the rat is the only one in which the liver is unable to methylate histamine *in vitro* (BROWN TOMCHICK & AXELROD 1959) In this species, however added histamine is methylated at a high rate in kidney tissue (BROWN, TOMCHICK & AXELROD 1959 NETTER, COHN & SHORE 1961 AZIZ 1961).

In sheep histamine is rapidly inactivated *in vitro* by kidney tissue (MCHENRY & GAVIN 1932), by the mucosa of the small intestines (SJAASTAD 1967a) and by liver tissue (SJAASTAD 1967b) In the two last mentioned tissues, the inactivation of histamine is almost completely abolished by aminoguanidine, a substance known to produce complete inhibition of histaminase (diamine oxidase) *in vitro* (SCHULER 1952 WATON 1956) Methylation does not seem to take place to any considerable extent. The

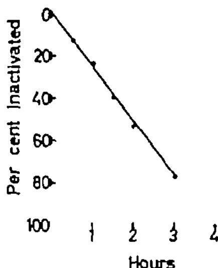


Fig. 2. Inactivation of histamine in kidney homogenate. Amount of tissue 200 mg Initial amounts of histamine: 400 µg. Final volume 4 1 ml. Each value is the mean of two incubations.

The histamine inactivating capacity of kidney tissue

In 27 specimens of kidney cortex from different sheep, the histamine inactivating capacity was 429 ± 102 (s) µg/g/hr (table 1) The inactivation of histamine in kidney homogenate (specimens from 4 animals) did not deviate much from the inactivation in kidney slices from the same animals (441 and 417 µg/g/hr., respectively)

Table 1

Inactivation of histamine in slices of kidney tissue.
Effect of aminoguanidine (10⁻ M).
Incubation period 2 hours. Histamine dded experiments with aminoguanidine 40 µg histamine diphosphate (HDP), experiments without aminoguanidine 400 µg HDP

Inactivation of histamine (µg HDP/g/hr)	
Without amin guanidine	With aminoguanidine
= 27	n = 12
429 ± 102 (s)	5 6 ± 1 0 (s)

Table 2

Effect of p-hydroxymercuribenzoate (PHMB) on the inactivation of histamine in kidney slices.
Initial amounts of histamine 20 µg. Incubation period: 2 hours.
Am unt f tissue 1 g.

Inactivation of histamine (µg/g/hr)	
Aminoguanidine 10^{-4} M	Aminoguanidine (10^{-4} M) + PHMB (10^{-3} M)
n = 8	n = 8
4.5 ± 0.5 (a)	4.2 ± 0.8 ( )

Effect of aminoguanidine

When kidney slices were incubated with 40 µg of histamine (10 µg/ml) in the presence of aminoguanidine, the rate of histamine inactivation was only 5 6 ± 1 0 µg/g/hr (table 1). This rate was not markedly increased when the concentration of histamine was increased to 25 µg/ml (4 expts.) Moreover the decrease in the concentration of histamine during the incubation period was then too small for accurate determination (average 12 %)

Effect of p-Hydroxymercuribenzoate (PHMB)

A small degree of inhibition by PHMB would not be easily detected in kidney tissue inactivating histamine at a high rate by pathways not affected by this substance. The inactivation of histamine in aminoguanidine-treated kidney slices was therefore compared with samples to which both aminoguanidine and PHMB had been added. No significant difference in the rate of histamine inactivation was found (table 2) Gassing with N_2 also abolished the greater part of the histamine inactivation in kidney tissue.

Discussion

The values for the histamine inactivating capacity of kidney tissue of sheep found in the present study (429 ± 102 µg histamine-diphosphate/g/hr) agree well with those obtained by McHenry & Gavin (1932) (369 µg histamine diphosphate/g/hr) The capacity of kidney tissue of sheep to inactivate histamine *in vitro* is thus about 3 times larger than that of liver tissue (Sjaastad 1967b) Cattle kidneys also inactivate histamine more rapidly *in vitro* than the liver (Holtz, Heise & Spreyer 1938).

Aminoguanidine almost completely abolished the inactivation of histamine in the present experiments (table 1) This suggests that primary

citrated plasma was used in which the kininase was inhibited by EDTA, but with the kallikrein-inactivators still present (BRISEID DYRUD & RINVIK 1967) The released kinin was assayed on the isolated rat uterus.

Technique

1 *Plasma substrate* The plasma substrate 1 described by BRISEID, DYRUD & RINVIK (1967) was used. In the present work each substrate specimen consisted of mixture of equal volumes of substrates obtained from 3 males in the age range of 30 to 57 years. The substrat was stored at $-20°$ as 1 ml samples corresponding t 0.87 ml plasma in 5 ml siliconized test tubes.

2. *Standard and test kallikrein. Padutin R* ® (hog pancreas kallikrein). In ampoules of 10 biol. units, Bayer A.G Leverkusen, Germany

3 *Rat uterus.* Virgin rats weighing 140–190 g were injected with stilboestrol (10 μg/100 g) 18–24 hours before use. Uteri were suspended in de Jalon's solution in 3 ml bath at $29° \pm 0.1$ and were usually used soon after the animal' death, but occasionally stored at 4 for one or two days. The muscle was attached to an isotonic frontal writing lever yielding magnification of about 5. The load was usually 1.2 g (range 0.8–1 4 g). A dose cycle of 4 minutes with 50 to 75 seconds contact and 4 washes was used.

4. *Preliminary bracketing assay"* T 3 one ml samples of the substrate, standard padutin was added with dose-ratio 1 0.7 0.5 (for the batch f padutin used 0.50, 0.35 and 0.25 units/ml plasma) and to 1 sample an amount of test padutin preparation was added expected to fall within the standard range mentioned. This was then diluted with saline and incubated in siliconized bottles as stated below under Method" The activity of the unknown kallikrein preparation was roughly estimated in "bracketing assays" with the standard and test solutions diluted to the same extent, and thus with a standard dose-ratio of 1 0.7

5 *Method.* To 2 one ml samples of plasma substrate standard padutin was added with dose-ratio 1 0.7 (for the batch of padutin used 0.40 and 0.28 units/ml). To 2 other samples test padutin was dded in amounts that according to the preliminary assay corresponded to the standard doses. One substrate sample served as blind test. All samples were diluted to 5.00 ml with saline and incubated in a waterbath at 37° in siliconized flasks. After 60 minutes 1 ml samples were withdrawn, 2.5 ml of saline were added and the mixtures heated in boiling waterbath for 5 minutes. The solutions were assayed at once or kept at 4° for assay within 24 hours. T obtain the same concentration of plasma, all the tests were equally diluted with de Jalon's solution.

The assay was carried out as (2 + 2) assay with 6 series of 4 randomized doses. Th individual contractions were measured to the nearest mm and the statistical procedure described in the Nordic Pharmacopoeia was used for the calculation of results.

Comments on the Technique

1 *Plasma substrate* The properties of the substrate used were discussed in the paper of BRISEID, DYRUD & RINVIK (1967), who found that the concentration of EDTA 2Na used (4 mg/ml of citrated plasma) completely inhibited the kininase present, while the inhibitors of kallikreins seemed not to be significantly influenced. Padutin was only slightly inhibited by EDTA.

HORTON (1959) preferred dog's plasma to human plasma since the latter rapidly formed kinin on contact with glass. In our experiments the use of siliconized test tubes prevented such an activation. The assay of the blind tests showed an activity which regularly amounted to about 10% of the releasable activity. Experiments carried out with incubation of almost undiluted substrate 1 specimens resulted in a decrease in "blind" activity to roughly 5%, indicating that dilution and not surface contact was responsible. The control incubates were not taken into account in the calculations of the assay results.

HORTON (1959) emphasized the significance of destroying kallikrein inhibitors, which would otherwise gradually slow down the rate of the release process. The removal of such influencing factors might improve the precision of the method, but as regards the specificity of a method intended for the assay of a kallikrein preparation used in therapy it seemed in principle advantageous to use a substrate as "genuine" as possible and preferably prepared from human plasma.

2. *Standard preparation.* Bradykinin was used as standard substance during the development of the method, for example in the establishment of kallikrein dose time effects (fig. 2). It proved necessary, however, to use kallikrein (or rather kinins liberated in a standard plasma by kallikrein) as standard in the assays. Determinations carried out on the same plasma substrate with bradykinin as standard usually gave results in a narrow range, but sometimes markedly deviating values were obtained. This must indicate that the different uteri did not react in a similar manner to bradykinin and to the released mixture of kinins.

3. *Method. Concentration effect curves.* The plasma substrates used for the experiments demonstrated in fig. 1 were both found to release 5.5 μg/ml fresh plasma of kinin calculated as bradykinin, when assayed by the trypsin method of RINVIK, DYRUD & BRISEID (1966) or by the acetone (plasma kallikrein) method described by BRISEID, DYRUD & RINVIK (1967). Fig. 1 shows that the maximum amounts of kinin liberated by padutin in the 2 substrate specimens were 3.9 μg and 4.6 μg/ml plasma, or on an average 77% of the theoretical value. It has previously been observed for a less "genuine" plasma substrate (pH 2 for 10 minutes at 37°) that padutin released about 70% of the total kinin present (BRISEID, JENSEN, DYRUD & RINVIK 1966). In specimens of this plasma substrate, the kallikrein incubation had to be followed by incubation with leucine aminopeptidase in order to convert small amounts of kallidin (or kallidin-like peptides) to bradykinin before assay on the uterus. In the present substrate no increases in activity were recorded on further incubation with leucine aminopeptidase.

Fig. 1 shows that for both substrate specimens used, about 2 units of

Table 2

Release of kinin in human plasma substrates. Insignificant inhibition by EDTA of hog pancreas kallikrein (padutin) and of plasma kallikrein.

Plasma substrate 1: EDTA 2 Na, 4 mg/ml citrated plasma.
– 1 + EDTA – 8 – – –

For details see text.

Releasing agent		Plasma substrate	μg kinin/ml plasma after incubation for			
			hours			
			5	7	9	24
Acetone (plasma kallikrein)		1	2.2	3.1	3.9	5.4
		1 + EDTA	1.9	3.1	3.7	5.4
			minutes			
			7.5	15	30	60
Padutin (hog pancreas kallikrein)	0.35 u/ml plasma	1	1.2	1.4	1.7	2.2
		1 + EDTA	1.2	1.3	1.4	1.7
	0.70 u/ml plasma	1	1.9	2.4	2.6	2.6
		1 + EDTA	1.7	2.2	2.4	2.4

strates 1, 2, 3, and 4 shown in table 1 (plasma specimen 2). In these experiments we incubated with a concentration of trypsin just high enough to cause full kinin release in the pH 2 treated substrates 2 and 4. It can be seen that the extent of release in the pH 5-treated substrate 3 was only slightly lower.

The addition of EDTA to substrates 2 and 4 in the trypsin experiments demonstrated weak inhibition by EDTA of trypsin as releasing agent, similar to that shown against the glandular kallikrein.

Table 2 shows experiments carried out in order to obtain more direct evidence of the inhibition by EDTA of hog pancreas kallikrein, and, if possible, also of plasma kallikrein. The table demonstrates that the rate of kinin release was slightly lower in plasma stabilized by 8 mg/ml than by 4 mg/ml of EDTA (substrate 1), when padutin was used as activating agent. The absence of effect of an increase in EDTA concentration in the acetone experiments might indicate that plasma kallikrein was less influenced than the glandular kallikrein, but it might equally well demonstrate other differences between the methods used.

The question whether EDTA inhibited the aminopeptidase of plasma in transforming kallidin (or other convertible kinins) to bradykinin (or other non-convertible kinins) in plasma substrate 1 is discussed below under Methods.

B. *Methods*

1. *Estimation of the rate of kinin release caused by acetone.* The activation of the kallikrein-kinin system of human plasma by acetone was first reported by FREY, KRAUT & WERLE (1950). Since then several research workers have used this method of initiating kinin

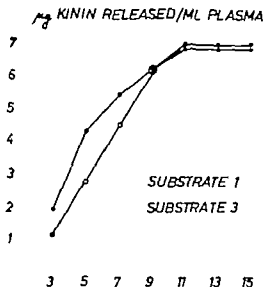


Fig. 1 Rate of release of kinin in human plasma substrates.
Activati g agent Acetone (plasma kallikrein).
Substrate 1 EDTA 2Na, 4 mg/ml citrated plasma.
Substrate 3 Hydrochloric acid to pH 5, EDTA 2N as in substrate 1
Kinin released calculated as μg bradykinin.
For details see text.

release in plasma, but without the intention of carrying out quantitative studies on the rate of the release process. MARGOLIS & BISHOP (1962) pointed out that acetone has a two-fold effect on plasma i.e. it slowly activates the H geman factor and it destroys inactivator of active kallikrein.

N ture of kinin released. Our experiments showed that under the conditions chosen acetone induced a complete exhaustion of plasma kininogen in the substrate suggested for the quantitative methods, substrate 1 and also in substrate 3 The fact that the maximum amounts of kinin obtained were the same as those determined by the kininogen method described previously (RINVIK, DYRUD & BRISEID 1966) using plasma substrate 2 and trypsin as releasing enzyme (T ble 4), is strong evidence that both methods give reliable estimates f the total kininogen content of plasma.

As the kinin released by trypsin in the acid heated substrate 2 was assumed to be identical with bradykinin (BRISEID JENSEN DYRUD & RINVIK 1966), the results of table 4 indicate either that the acetone-activated plasma kallikrein released bradykinin only or that the transf rming minopeptidases f plasma were not markedly inhibited by the actual acetone concentration. Model experiments carried out with synthetic kallidin and leucine aminopeptidase in buffer solution demonstrated some inhibition of the enzyme by an acetone concentration as low as 4 / (/v) nd complete inhibition at 10% (v/v). On the other hand, experiments with addition of kallidin to plasma substrate 1 and acetone activation as suggested for the method, demonstrated that complete conversion of kallidin to bradykinin did in fact tak place. These results leave open the question what kind of kinin was released by plasma kallikrein in the acetone method. The experiments showed that EDTA in the concentration used did not prevent complete transformation by the

Table 3

I Determination of the rate of kinin release caused by acetone.
II. Determination of the extent of kinin release caused by hog pancreas kallikrein (padutin).
Precision of the methods.

Plasma substrate 1 (see Table 1).
Maximum release by padutin obtained with 4 units/ml plasma.
For each method 12 separate substrate samples from the same plasma specimens were assayed at different times.
The plasma specimens used were not the same for the 2 methods.
For details see text.

Sample	I Time in hours corresponding to 50% kinin release caused by acetone	II Kinin release as % of maximum padutin units/ml plasma 0.35	 0.70
1	6.0	36	68
2	5.9	46	60
3	6.0	51	63
4	6.0	51	63
5	6.0	54	88
6	6.2	55	77
7	6.2	46	69
8	6.0	55	72
9	6.6	60	86
10	6.1	55	83
11	6.2	43	75
12	6.2	51	81
Mean	6.12	50.3	73.8
s.e.m.	0.05 (0.8%)	1.9 (3.8%)	2.7 (3.7%)

kallidin-converting enzymes of plasma. This does not mean that there was no inhibition at all.

Time-release curves. Fig. 1 shows 2 time-release curves based on the acetone activation technique. Both curves were drawn from results obtained with samples from the same plasma specimen, which for curve I had been stabilized with EDTA alone (substrate 1), while the citrated plasma used for curve II had in addition been incubated at pH 5 (substrate 3). It can be seen that the rate of release in the first hours was faster for substrate 3, in which inhibitors of kallikrein had been partly inactivated. After 9 hours, however the same amount of kinin had been released in both substrates, and after 11 hours both substrates were fully depleted of their kininogen. The time interval for 50% kinin release in this plasma specimen was about 6 hours for substrate 1 and 4 hours for substrate 3.

Reproducibility The precision of the acetone method is shown in table 3. Twelve different samples from the same plasma specimen (substrate 1) were assayed t different times. The amounts of kinin released after 5 and 7 hours, expressed as percentages of the

maximum mounts set free after 4 hours, were used to calculate the periods of time necessary for 50 % release.

It proved essential for the precision of the method to use well filled test tubes, which were stoppered during the incubation period. It was also found advisable not to withdraw more than 2 samples for assay from each test tube. If such precautions were not taken, evaporation of the acetone took place to a varying degree, thus decreasing the accuracy of the results.

Controls incubated for 24 hours without the addition of acetone showed activities of 0.8 μg/ml plasma or less, calculated as bradykinin, and were not taken into account in the assays.

2. *Estimation of the rate and the extent f release caused by padutin.* The effects of padutin in plasma substrates prepared by different procedures have been discussed previously (Briseid Jensen, Dyrud & Rinvik 1966)

a. *The time-effect relationship* of kinin release caused by padutin was examined for 2 concentrations of the enzyme, i.e. 0.35 and 0.70 units/ml plasma. It has been shown by Bieltvedt & Briseid (1967) that the time required for maximum release effect in substrate I increased with f lling enzyme concentrations, from bout 20 minutes for 2 units/ml plasma to about 60 minutes for 0 13 units/ml. We accordingly chose an incubation period of 60 minutes to obtai full kinin release. The padutin concentrations were fixed so as to bring about partial release (66 %) in the time interval 10 to 30 minutes. This was done with view to later use of the method for the detection of slower or faster rates of release in pathological plasma specimens.

As the log time release curves proved to be rectilinear the observation periods for partial releases were spaced logarithmically

b. *Th concentration-effect relationship* of kinin release caused by padutin was examined for the same 2 enzyme concentrations as those used for the estimation of the rate of kinin release, i.e. 0.35 and 0.70 units/ml plasma. In these experiments only 60-minute incubation results were used. Bieltvedt & Briseid (1967) found that the enzyme release curve as steepest for concentrations ranging from 0.. t 0.6 units/ml plasma and suggested a dose ratio of 1 0.7 (0.40 and 0.28 units/ml) for (2 + 2) assays. A padutin concentration of about 2 units/ml plasma was required for maximum release. In the present work it was found convenient to use somewhat higher concentrations of padutin, in order to utilize the results already provided for the simultaneous estimations of rates of release, and concentration of 4 units/ml was used to ensure maximum release of kinin.

R producibility The precision of padutin concentration-effect determinations is shown in table 3 Twelve different samples from the same plasma specimen (substrate I) were assayed at different times. Th amounts of kinin released by 0.35 and by 0.70 units/ml plasma after incubation for 60 minutes were expressed as percentages of the maximum amounts released after incubation with 4 units/ml.

Controls incubated for 60 minutes without the ddition of padutin showed activities ranging from 0.2 to 0.8 μg/ml plasma (most often 0.3–0.5 μg/ml) calculated as bradykinin, and were not taken into account in the assays.

Results

Table 4 shows the results of determinations of the rates of release of kinin caused by acetone (plasma kallikrein) in plasma specimens from 9 healthy males within the age range 25 to 57 years. The table also shows the

Table 4

Determination of plasma kininogen and of rate of release of kinin in 9 healthy males.

I. Substrate 2 (see Table 1)
II and III. Substrate 1 (see Table 1).
For details see text.

Subject number	I Kinin released/ml plasma as µg bradykinin: Trypsin method	II Kinin released/ml plasma as µg bradykinin: Acetone method	III Time in hours corresponding to 50 % kinin release: Acetone method
1	6.7	6.8	6.1
2	5.4	4.6	7.1
3	5.4	4.6	6.9
4	5.4	6.2	5.9
5	5.9	5.7	6.2
6	5.6	5.7	6.5
7	5.6	5.4	6.7
8	5.6	5.5	8.5
9	5.6	5.6	5.3
Mean	5.69	5.57	6.58
s.e.m.	0.14 (2.5 %)	0.23 (4.1 %)	0.30 (4.6 %)

results of determinations of plasma kininogen in the same men by the acetone method and by the trypsin method described by Rinvik, Dyrud & Briseid (1966)

The average amount of kininogen corresponded to a bradykinin release of 5.6 µg/ml fresh plasma when acetone was used as activating agent in substrate 1 (range 4.6 to 6.8 µg/ml). The average value for trypsin as releasing enzyme in the acid-treated substrate 2 was 5.7 µg/ml plasma (range 5.4 to 6.7 µg/ml) The results obtained by these 2 different methods thus agree very closely and they also correspond well with the mean value previously reported from determinations in 11 healthy males using the trypsin method and substrate 2, 5.5 µg/ml plasma (Rinvik, Dyrud & Briseid 1966)

The average time required for a release by acetone of 50 % of the total kininogen under the conditions used was 6.6 hours, range 5.3 to 8.5 hours.

Table 5 shows the results of determinations of the rates of release of kinin in plasma substrate 1 caused by padutin, and the table also demon-

Table 5

Determination of rate (I) and extent (II) of release of kinin in 9 healthy males.

Releasing agent Hog pancreas kallikrein (padutin).
Maximum release values obtained with 4 units/ml plasma.
Plasma substrate 1 (see Table 1)
For details see text.

Subject number	I. Time in minutes corresponding to 66% kinin release caused by padutin, units/ml plasma		II. Kinin release as % of maximum caused by padutin, units/ml plasma	
	0.35	0.70	0.35	0.70
1	15	13	33	42
2	16	12	63	72
3	15	8	67	86
4	33	15	100	100
5	19	23	48	78
6	13	11	50	69
7	39	23	34	47
8	19	15	35	54
9	17	13	63	69
Mean	20.7	14.8	54.8	68.6
s.e.m.	3.0 (14.5%)	1.7 (11.5%)	7.[illegible] (13.1%)	6.2 (9.0%)

strates the extent of release caused by this enzyme. The plasma specimens used originated from the same 9 men who were used for the determinations shown in table 4 Both the rate and the extent of kinin release were examined for 2 concentrations of padutin, i.e. 0.35 and 0.70 units/ml plasma.

Discussion

A plasma substrate appropriate for the estimation of the rate of release of kinin in human plasma should be "genuine" as far as possible. Such a demand is difficult to satisfy among other things because of the necessity of eliminating the kininase activity present. Even if the possibility is ignored that amounts and characteristics of factors involved in the release process might have altered, to some extent, during the preparation of the plasma substrate used several objections can be raised against an

acetone activation technique as a basis for the estimation of the rate of kinin release. It has been suggested that acetone acts through an activation of Hageman factor which in turn activates plasma kallikrein, but it has also been pointed out that acetone at the same time will inactivate inhibitors of plasma kallikrein (MARGOLIS & BISHOP 1962 EISEN 1963). Any failure in the kallikrein inhibition system of plasma will accordingly only be recognized by the acetone method if the rate of inactivation of the inhibitors by acetone is significantly slower than the kallikrein activation process.

To increase the possibility of detecting deficiencies in the kallikrein inhibitors of plasma, in addition to the acetone technique, we developed a method based on the release of kinin by hog pancreas kallikrein (padutin) Like other glandular kallikreins, padutin acts directly on the kininogen to release kinin, and deviations from the normal rate of release observed, should indicate failures in the kallikrein inhibiting system. It is then assumed that the factors inhibiting plasma kallikrein and hog pancreas kallikrein are essentially the same.

The fact that the maximum amounts of kinin obtained by the acetone method (plasma kallikrein) were found to be almost the same as those registered by the trypsin method (RINVIK, DYRUD & BRISEID 1966) is strong evidence that both methods give reliable results for total kininogen. If a loss of kinin should nevertheless have taken place it must have occurred at a stage of the substrate preparation procedure prior to the acidification or EDTA treatment of the centrifuged citrated plasma.

The good correlation between the 2 methods also show that the amounts of the plasma factors involved in the release process are in excess of the amount of kininogen present.

The amounts of kinin released by padutin were not only used for the estimation of the *rate* of the release process, but also for estimating the *extent* of the enzyme effect, or in other words, the concentrations required for a certain release effect. For example, a low activity level of the inhibitors of kallikrein would be expected to cause both an increased rate of release, and a higher percentage of kinin released than that normal at a certain padutin concentration. A high rate of kinin release together with a normal extent of release, on the other hand, might indicate anomalies in the types of kininogen present.

The combined use of the methods described in our work and the previously published methods for the determination of plasma kininogen and plasma kininase (RINVIK, DYRUD & BRISEID 1966) are of interest in the study of the kinin activity level of plasma under normal and patholog ical conditions.

Summary

Methods have been described for the estimation of the rate and also the extent of release of kinin in human citrated plasma stabilized by EDTA 2 Na, 4 mg/ml plasma. The kinin released was assayed on the isolated rat uterus with bradykinin as standard.

The estimation of the *rate* of the release process was based on determinations of kinin set free at different time intervals by (1) acetone (through plasma kallikrein) and by (2) padutin (hog pancreas kallikrein). The rate of the release process was given as the period of time required for a release of 50% (acetone) or of 66% (padutin) of the maximum amount obtained after a prolonged period.

The estimation of the *extent* of kinin release was based on the determination of the maximum amount of kinin set free at a certain padutin concentration in percentage of the maximum amount released by excess of enzyme.

The methods were used to estimate the release process in plasma specimens of 9 healthy men Under the conditions used (10% v/v of acetone and 20% v/v of citrated plasma in saline at 37°) the time required for a 50% release by acetone was 6.6 hours (s.e.m. 4 6%) The periods of time required for 66% release by padutin were 20 7 minutes (s.e.m. 14.5%) and 14 8 minutes (s.e.m. 11.5%) respectively for concentrations of the enzyme of 0 35 and 0 70 units/ml plasma. The same 2 padutin concentrations caused releases of kinin of 54.8% (s.e.m. 13 1%) and 68 6% (s.e.m. 9.0%) respectively of the maximum.

The reproducibility of the methods was measured by determinations of 12 separate samples from the same substrate specimen at different times. The s.e.m. for the acetone method was 0 8% and for the padutin method measuring the extent of release, 3 8 and 3 7% for 0 35 and 0.70 units respectively

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Table 1

The exact chemical composition of kerosene)
used in the study

Paraffins/Naphthenes (no unsaturates)	80.00 %	
Aromatics	20.00 %	
Sulphur	00.25 %	maximum by weight
Mercaptans	00.005 %	maximum by weight

) Courtesy-General Manager Esso Standard Inc., U.S.A.

Methods

The kerosene used in these studies on experimental kerosene poisoning was obtained from the Burmah-Shell Refineries, Caltex (California-Texas) Oil Refineries and the Esso Oil Refining Ltd (USA), which are the three main brands available in India. The source of these kerosenes is from the Middle-East, which is also the major source of supply for both America and Europe. The results of the experiments were bsolutely uniform and constant with all the three brands of kerosene used since there was no difference in composition or purity between any of the three brands. The chemical composition is given in table 1; the degree of purity being 97 ± 0.5 %.

Swiss albino mice, Belgian rabbits and dogs were used in the experiments. *Swiss mice* weighing 30 g were used and their respiratory rates, neuromuscular strength (as determined by the inclined plane method) were measured. The animals to be treated were each given 1 ml of filtered kerosene orally while the animals set part as controls were given the same amount of distilled water. The animals were kept under close observation and twenty-four hours later a second dose of 1 ml of kerosene was given to each mouse in the treated group, while the controls received 1 ml of distilled water.

In the second set of experiments four groups of dogs, each consisting of four dogs, each dog weighing 7–8 kg were used. Kerosene was administered intraperitoneally in a dose of 50 ml/kg body weight. The various clinical changes were recorded. Liver function tests were carried out using the bromsulphalein liver function test. The blood sugar levels were estimated, every two hours, by the Folin-Wu method. Later necropsy was done on the animals.

In the third series of experiments, *Belgian rabbits* weighing bout 1.5 kg were given kerosene orally in dose of 70 ml/kg body weight. The blood sugar levels were determined by the micro-method. Both macroscopic and microscopic examinations were performed at necropsy

Chemical tests for the detectio of kerosene or its fractions in blood or urine could not be done, since kerosene is heterogenous mixture of various hydrocarbons and no specific chemical test is known (SACHANEN 1945).

Results

1 *Mice*

Mice treated orally with a single dose of kerosene became drowsy within 12 to 15 minutes. There was dyspnoea and tachypnoea, drowsiness becoming very pronounced by the second hour The neuromuscular-

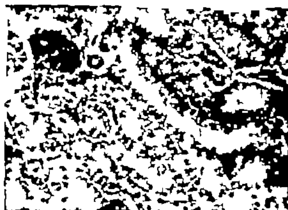


Fig. 1 Kidney of albino mice treated with kerosene showing changes of *cloudy degeneration* with fraying of the cell margins.

strength as determined by the 'inclined plane method' diminished significantly as compared with the controls. Four hours after treatment, the mice recovered but their coats were still shaggy and smelled of kerosene, and the anogenital region was stained with kerosene. The animals gradually became normal in movement and feeding habits.

Twenty-four hours later when a second dose of kerosene was administered, the same signs were seen. However drowsiness became more pronounced, and by the fifth hour the animals became comatose. All the treated mice died after 8 to 10 hours. The experiments were repeated twice and the same results were observed.

Autopsy findings

The *stomach* wall appeared thin and contained the previous meal mixed with kerosene. The mucosa was rough in appearance. Histological studies indicated that the serous and the muscular layers were normal. The lamina propria was also intact, but the mucosa had disintegrated though a few shreds were seen. The submucosal area appeared swollen.

The external appearance and the cut surfaces of the *kidney* appeared normal. Histological studies of the renal tubules showed cloudy degeneration. The glomeruli and the pelvis were congested (fig. 1). On macroscopic and microscopic examination the *spleen* was normal.

The external appearance and microtome sections showed the *brain* to be normal. Histological studies showed that the meninges were hyperaemic and congested. However the brain tissue appeared normal. Externally the *lungs* and the *pleurae* were normal. The cut surface was hyperaemic. The trachea and the hilar glands were normal. *Histological*

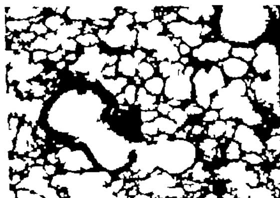


Fig. 2. Lung of animal treated with kerosene showing *no pneumonia* or any inflammatory cells, but only congestion.

examination showed that the main blood vessels were dilated and that the alveolar walls were hyperaemic. Certain alveoli in the basal lobes contained a few RBCs. There was absolutely no evidence of cellular exudation and no cells of inflammation were observed anywhere in the lungs or pleura. The bronchi and the bronchioles were normal and showed completely normal mucosa (fig. 2).

The *liver* was enlarged, pale in appearance, with conspicuous yellow patches scattered on the surface. The liver tissue smelled of kerosene and when placed in formalin, kerosene exuded out of the tissue, forming a thin layer on the surface. Histological examination showed that the central veins were normal. The centri-lobular zone appeared normal, but the intermediate zone showed vacuolation with cloudy degeneration. The periportal zone showed similar vacuolation, but to a lesser extent (figs. 3 and 4)



Fig. 3. Liver of albino mice treated with kerosene showing *fatty degeneration* and extensive cloudy swelling.

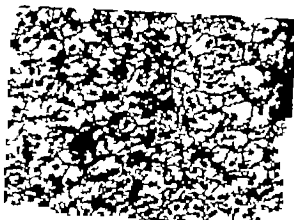


Fig. 4 Liver of animal treated with kerosene showing a severe degree of *fatty degeneration*, with complete replacement of the cellular cytoplasm with lipoid material.

2. *Dogs*

Within 15 minutes to one hour after intraperitoneal administration of kerosene (50 mg/kg) the animals became restless and excited. After one hour the typical symptoms noticed in mice were also seen here. Tachycardia and tachypnoea with a rise in body temperature to 39.3 occurred. The breath smelled of kerosene. Later the treated animals appeared sedated and drowsy Their urine also smelled of kerosene. Though they were dyspnoeic, they were still conscious. After six hours, the dyspnoea increased and tachycardia persisted. The corneal and conjunctival reflexes were present. However the dogs did not respond to pain sensations like pin pricks. On being forcibly made to stand, the animals staggered. By the seventh or eighth hour two dogs showed mild tremors of the extremities. Five dogs had convulsions which persisted only for a short period. The remaining dogs had no convulsions and gradually became comatose. This condition lasted for a few hours, eventually ending in the death of the animals.

Autopsy findings

The *lungs* appeared congested, but no fluid could be expressed from the tracheobronchial tree. Microscopically the alveoli and the bronchi were normal and the blood vessels were dilated. No inflammatory cells or any exudate could be seen.

The *heart* appeared normal and this was confirmed by histological examination.

The *meninges* showed congestion but the *brain* was normal both macroscopically and microscopically

Table 2

The results of the bromsulphalein liver function test in dogs after the administration of kerosene (In terms of % retention)

Group	0 hr	2 hr	4 hr	6 hr.	8 hr
A	4.1	19 1	50.9	70.4	88.0
B	7.9	22.7	58.9	63.0	80.8
C	0.7	17.4	37.0	54 7	74.7
D	5.6	29.0	48.7	60.8	78.3

Table 3

The average blood sugar levels in mg/ml of each group of dogs after the administration of kerosene.

Group	0 hr	2 hr	4 hr.	6 hr	8 hr
A	1 100	0.913	0.700	0.679	0.437
B	1 045	1.001	0.814	0.593	0.528
C	1 172	0.908	0.803	0.532	0.402
D	0.987	0.901	0.656	0.509	0.504

No lesions were seen superficially in the *kidneys* but histological examination showed severe cloudy degeneration. The *urinary bladder* was severely congested and denuded in certain areas.

The *liver* appeared congested. Histological examination showed cloudy degeneration of the parenchyma with intense hyperaemia. The liver func tion was affected and ultimately severely impaired as seen in the bromsulphalem tests (table 2) The blood sugar level fell and this gradually led to hypoglycemia (table 3)

The *gastro-intestinal tract* (including stomach, small and large intest ines) showed on microscopic examination that the mucosa was oedema tous and denuded in patches

3 *Rabbits*

The rabbits showed tachycardia and hyperpyrexia up to 38 6° by the third hour Their coats were shaggy and stained with kerosene. Signs of tachypnoea persisted throughout the day After 19 hours the dyspnoea

Table 4

The average blood sugar levels in mg/ml in each group of Belgian rabbits after the administration of kerosene.

Group	0 hr	2 hr	4 hr	6 hr	8 hr
A	1.248	1.074	0.970	0.678	0.594
B	1.071	1.007	0.875	0.734	0.637
C	1 112	0.986	0.732	0.640	0.539
D	1.048	0.975	0.787	0.721	0.598
E	1.025	1.014	0.741	0.567	0.437

had increased. The corneal responses were present and the animal responded to pain stimuli. By 22 hours the dyspnoea had increased further and the animal showed loss of righting reflex. The extensor tone appeared to be increased as the rabbits showed neck rigidity and complete extension of the extremities The animals went into convulsions and died without going into coma. As in dogs there was a progressive type of hypoglycemia (table 4)

Autopsy findings

Macroscopic examination of the *liver* showed yellow areas on the surface. Cut sections revealed areas of focal necrosis in places where the tissues had been completely destroyed. Microscopically there was complete tissue destruction surrounded by necrotic parenchyma and inflammatory cells. At other sites fatty degeneration was noted (fig. 5).



Fig. 5. Liver of Belgian rabbits treated with kerosene showing gross areas of *focal necrosis* with tissue destruction leaving cyst-like spaces.



Fig. 6. Stomach of animal treated with kerosene showing areas of extensive ulceration, with cellular infiltration, reaching the muscular layer

Macroscopic and microscopic examination of the *lung* parenchyma and bronchioles revealed no change except congestion. Despite careful scrutiny no inflammatory cells of any kind were seen.

In the *brain* the meninges were hyperaemic, but no inflammatory cells were seen.

The *kidney* tubules showed cloudy degeneration with fraying of cell margins. The glomeruli showed severe congestion

Macroscopically the mucosa of the *stomach* was oedematous, congested and desquamated in certain places. Histological examination showed destruction of the mucosal cells at places reaching as deep as the muscular coat (fig. 6). Numerous polymorphs and other inflammatory cells were visible

Microscopic examination of the *small intestines* showed tissue congestion with desquamation of mucosal cells (fig. 7)



Fig. 7 Small intestine of Belgian rabbits treated with kerosene showing gross erosion and desquamation of the mucosa.

No lesions were visible either macroscopically or histologically in *heart and spleen*

Discussion

The current view on kerosene poisoning is that when it is ingested, it becomes volatilized and this vaporized kerosene is aspirated by the lungs causing pneumonia. The site of action of kerosene poisoning is believed to be the lungs and death results from aspiration pneumonia (WARING 1933). DEICHMANN *et al* (1944) however showed the two-fold nature of absorption of kerosene through the gastro-intestinal tract and excretion in the lungs, the resulting death being attributed to kerosene pneumonia. The present studies however show that both these previous views do not explain the true mode of action and previous concepts need to be changed.

DEICHMANN *et al* showed that kerosene is absorbed through the gastro-intestinal tract, which has been confirmed in our studies. On oral administration of kerosene no signs of pneumonia – which is essentially an inflammatory reaction were seen, and there was complete absence of any inflammatory cells or exudate The lungs were merely hyperaemic and congested, which does not indicate pneumonia. The liver on the other hand, showed a definite pathological picture of acute vacuolation as seen in the mice and in the multiple areas of necrosis and cavitation in the rabbits

In dogs the liver showed cloudy degeneration. The physiological dysfunctioning of the liver is indicated by the marked bromsulphalein retention, even up to 80 / in the liver tissue. The clinical deterioration of the dog following kerosene administration runs parallel with the failing function of the liver as determined by the bromsulphalein liver function tests (see table 2).

The kerosenes used in our studies were those obtained from the Burmah-Shell Refineries Caltex Oil Refineries, and Esso Standard Inc., which *are the three major sources available in India and America* and Europe and the clinical cases of poisoning are due to these agents hence our experiments accurately reflected the clinical findings. There is no question that any particular sample of kerosene contained a substance toxic to the liver since liver damage resulted from the administration of all brands of kerosene.

The acute fall of the blood sugar level is another index of the physiological destruction of the liver The peak of hypoglycemia appears simultaneously with the onset of convulsions and coma, indicating that these reactions are not due to the action of kerosene on the brain centres (PRICE 1932) but rather due to hypoglycemia. The hypoglycemia seen

following kerosene administration is not due to fasting as is shown by the fact that the fasting blood sugar levels in rabbits is 85 mg% (SPECTOR 1964) and 95 mg / ± 10, according to our estimations in the controls. Since the animals used in the experiments were not fasted overnight, fasting for 8 hours only would not produce such a severe fall of up to 40–45 mg / moreover the controls which had levels of 95 mg / ± 10 were not fed during the experiment.

The intraperitoneal route was adapted in the dogs as suggested by RICHARDSON & PRATT THOMAS (1951), and BRUNNER, ROVSING & WULF (1964) who state that dogs are unsuitable for the oral administration of kerosene, since they vomit it out in a very short time. We observed this in our first experiments on dogs. The above investigators even had to ligate the oesophagus to prevent the spontaneous vomiting in dogs. This vomiting confuses the real picture, as it gives rise to secondary complica tions of aspiration pneumonia. We may add that in clinical cases of kerosene poisoning spontaneous vomiting is not seen in man, and drastic procedures have to be used to induce emesis (BARBOUR 1926 PRICE 1932 and our hospital cases). DEICHMAN *et al* (1944) used the intraperitoneal route in order to prevent the possibility of intrapulmonary aspiration. He even tried the intravenous route in rabbits.

The pathognomonic cause of death in kerosene poisoning is acute liver cell failure rather than kerosene pneumonia which may develop as a secondary complication. The major organ affected by kerosene is therefore the liver Consequently any therapy for treating kerosene poisoning in children should be directed at reducing the damage to the liver including liver transfusion

Summary

Cases of death of children following accidental oral ingestion of kerosene have frequently been reported in India and western countries. Previous workers had indicated that death was the result of aspiration of kerosene into the lungs. Experiments carried out by us on mice, rabbits and dogs showed that the primary cause of death following oral ingestion of kerosene is impairment of liver function and resultant hypoglycemia leading to convulsions and coma. No inflammatory reac tions of any kind were seen in the lungs except hyperaemia.

Acknowledgements

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Effect of Ethanol on the Growth and on the Liver and Brain Mitochondrial Functions of the Offspring of Rats

By

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(Received August 27 1966)

Ethanol in 15 / ethanol solution, given to rats over a long period, influences liver mitochondrial oxidation and adenosine triphosphatase activity (KIESSLING & TILANDER 1961 & 1963), the thiamin and thiamin diphosphate level (KIESSLING & TILANDER 1960) and the size and shape of the liver mitochondria (KIESSLING & TOBÉ 1964 KIESSLING & PILSTRÖM 1966a & 1966b) Brain mitochondria from ethanol-treated rats do not show any change in respiration (KIESSLING & TILANDER 1963).

In the present work the effect of ethanol on the foetus has been studied, the drug being supplied to the foetus via its mother The birth weight and growth of the offspring as well as the respiration and oxidative phosphorylation of mitochondria from the liver and brain of the offspring have been investigated.

Experimental

Three months old female rats of Wistar origin were divided into two groups. One group was given 15 % (v/v) solution of ethanol as the only drinking fluid, and the other group water and sucrose solution, isocaloric with the ethanol consumed by the rats in the first group. Each rat was kept in a separate cage and had free access to solid food, adequately balanced as regards carbohydrates, lipids, proteins, vitamins and minerals. Two to four weeks later the females are paired, the same male being used for both a female from the ethanol-treated group and female from the control group.

After parturition the young were weighed as soon as possible and the first six born from each litter used in the experiments. The remaining young were weighed every day for fortnight.

The remaining young from tw control litters were taken from their mothers, and the young from the corresponding ethanol-drinking females allowed to suckle the control females instead of their actual mothers. The young were weighed every day

The six young from each litter taken for the experiment were decapitated and the brains and livers removed as fast as possible and chilled in an ice-cold 0.25 M sucrose solution. Six brains weighed bout 1 7 g and six livers about 1.2 g. The two tissues were cut with a pair of scissors and as much blood as possible was rinsed off with ice-cold 0.25 M sucrose. Finally the livers and the brains were homogenized in a Potter Elvehjelm all-glass homogenizer

Liver mitochondria were prepared in 0.25 M sucrose, according to SCHNEIDER & HOGEBOOM (1950), and brain mitochondria in 0.25 M sucrose with ATP (60 mg per 100 ml), as described by JÖBSIS (1963). During the whole preparation procedure the temperature of the solutions was kept at between 0 and +4

The mitochondrial respiration was determined by the conventional Warburg method with air as the gas phase and with 0.2 ml 2 M KOH in the centre well. Each vessel contained mitochondria from 250 mg of tissue, together with 3 μmoles ATP 10 μmoles Mg^{2+} 45 μmoles glucose, 63 μmoles phosphate buffer (pH 7.4), 0.5 mg hexokinase (Sigma Grade III) and 15 μmoles substrate in a final vol me of 1 0 ml. Pyruvate (with 10% malate), glutamate or β-hydroxybutyrate were used as substrates. I the vessels with brain mitochondria and pyruvate as the substrate, NAD^+ was added to a final concentration of 0.25 mg/ml.

The measurements were performed at 30° and lasted for 20 min. for the liver mitochondria nd for 25 min. for the brain mitochondria, including 5 min. for thermoequilibration.

The phosphorylation was determined by the method of isotope distribution, according to LINDBERG & ERNSTER (1955) and the protein content f the mitochondria by the biuret reaction, as described by CLELAND & SLATER (1953).

Results

Birth weight and growth

The birth weights of the offspring of ethanol-treated females and control females are shown in table 1 Those from the control females had an 8 / higher birth weight than those from the ethanol-drinking females. The difference is statistically significant with $p < 0\ 001$ The mean litter sizes of the two groups are also given in table 1 The mean size of the

Table 1

Body weight and litter size

The mean body weight nd litter size of young of female rats given 15% (v/v) ethanol solution as drinking fluid and also of control females.

	Control young	Ethanol-treated young
Mean body weight (g)	5 43	5.02
Number of young	77	125
Standard deviation	0.44	0.57
	$t = 5\ 74$	$p < 0.001$
Litter size (young/litter)	9 1	10.4
Number of litters	9	12
Standard deviation	2.31	1 73
	$t = 1\ 40$	$p = 0.20$

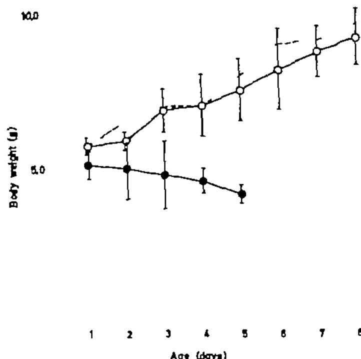


Fig. 1 The change in postnatal body weight with time.
The investigation was carried out on 18 control young (O——O) and 36 (1st day) and 11 (5th day) ethanol-treated young (●——●). The standard deviation for each value is indicated in the figure.

The dotted line shows the mean weights of 7 young borne by ethanol-treated females and suckled by control females.

litters from ethanol-treated females was 14 % greater than that of the litters from the control females. This difference is, however not statistically significant.

The growth rate, obtained by daily weighing of the remaining young, during the fortnight after parturition is shown in fig. 1 In contrast to the young from the control females, those from the ethanol-drinking females lost weight and the mortality in this group rose until, after five days, there were not enough young left for further studies. The young from the control females gained weight continuously and had doubled their birth weight after ten days.

The young born from ethanol-treated females but suckled by control females grew like the control young, as is seen in fig. 1 No difference in mortality was found in this group

Respiration and oxidative phosphorylation of mitochondria from liver and brain

Table 2 shows the respiration of liver and brain mitochondria from the young belonging to the ethanol treated group and also those from the controls. A slight decrease in mitochondrial respiration was found after ethanol treatment with pyruvate or glutamate as substrates, but these differences were not statistically significant. When β-hydroxybutyrate was used as substrate, the mitochondrial respiration did not differ between the two groups.

The oxidative phosphorylation was low in mitochondria from both the neonatal liver and brain with all the substrates tested (table 2). The low phosphorylation when β-hydroxybutyrate was used as substrate, is remarkable

Table 2

Respiration and phosphorylation of liver and brain mitochondria from neonatal rats (E) young borne by females given ethanol, (C) young borne by control females. For details of preparation and incubation procedures, see Experimental.

Tissue	Substrate	Treatment	Respiration (µatom O_2/ hour × 10 mg protein)	No. of experiments	S.D	*t* for C − E	P:O ratio
Liver	Pyruvate	C	17.11	10	3.21	1.07	2.35
		E	15.48	10	3.59		2.28
	Glutamate	C	16.68	9	3.03	0.96	2.12
		E	15.21	10	3.62		2.36
	β-OH-butyrate	C	6.42	7	1.79	0.37	1.69
		E	6.02	7	1.88		2.05
Brain	Pyruvate	C	17.81	10	4.81	1.22	2.49
		E	15.61	11	3.17		2.40
	Glutamate	C	15.16	10	4.47	1.23	2.25
		E	13.11	9	2.67		2.41
	β-OH-butyrate	C	5.85	7	1.86	0.34	1.52
		E	5.51	8	2.02		1.92

Discussion

Altogether the effect of ethanol on the foetus has been very little studied, as has already been pointed out by BAKER (1960) in a review of drug effects on the foetus. The foetal cells are very dynamic, with rapid and marked changes in the enzyme activities of both soluble or structural origin (STRITTMATTER & STRITTMATTER 1961 BURCH *et al.* 1963 STEVENS 1962) The particle bound oxidative enzyme systems show increases in activities during certain stages of the embryonic development, and relative changes in mitochondrial enzyme activities have been studied and taken as an indication of the differentiation of mitochondria during development (BRAND & MAHLER 1963 STRITTMATTER 1963)

It is therefore reasonable to expect that ethanol, which slowly affects the subcellular functions in the adult animal (KIESSLING & TILANDER 1961 & 1963 KIESSLING & PILSTRÖM 1966a), may interfere with these processes more rapidly during the pre and neonatal period.

Ethanol passes through the placenta and reaches the foetus, as demonstrated by OLOW (1923a). He also showed that during acute ethanol intoxication, ethanol was found in the same concentrations in breast milk and in blood (OLOW 1923b) It should thus be possible to supply ethanol to the offspring during gestation and the suckling periods by giving the mother ethanol solution as drinking fluid.

Birth weight growth and litter size

The effect of ethanol on the birth weight of offspring was investigated by BLUHM (1930) in mice. She found no difference in the birth weights between young from ethanol-treated females and controls. Our material shows a significant difference in birth weight (table 1). It has, however been demonstrated that young from a large litter have a lower mean birth weight than those from a small litter (IBSEN 1928). The ethanol-drinking females gave birth to a larger number of young per litter than did the controls (table 1). This fact may be responsible for the difference observed. Hence, it cannot be concluded that the ethanol treatment causes any alterations in birth weight.

In our study ethanol-treated females and controls gave birth to litters of different mean sizes. As can be seen in table 1 however the standard deviations and the number of experiments do not allow the conclusion that ethanol generally alters the litter size.

BLUHM (1930) found a greater mortality in the young from ethanol treated mothers than in controls. This is in agreement with our own results, as the mortality in the alcohol-treated group was so great that

only very few young lived longer than one week. The mean body weight decreased continuously from birth to death while the control young grew normally (fig. 1). This may be explained by some observations of FUCHS & WAGNER (1963). They found that ethanol inhibits the release of oxytocin from the pituitary gland when suckling acts as a stimulus. Oxytocin is essential for milk ejection. An inhibition of the release of this hormone would thus result in no milk reaching the suckling cubs.

The results of FUCHS & WAGNER (1963) are supported by two of our observations. 1) The stomachs of the ethanol-treated offspring contained no or very little milk, while those of the control offspring were always filled with milk. 2) When young borne by ethanol-treated females were allowed to suckle a control female, their body weights rose normally as indicated by the dotted line in fig. 1.

The loss of body weight and the high mortality thus seem to be the result of starvation, due to impaired milk ejection caused by ethanol.

Respiration and oxidative phosphorylation of mitochondria from liver and brain

Table 2 shows that treatment of the mother with ethanol during gestation did not alter the normal development of the foetal liver and brain mitochondria either in their capacity to oxidize pyruvate, glutamate and β-hydroxybutyrate or in their oxidative phosphorylation.

Thus ethanol continuously supplied to rat foetuses during prenatal life does not cause a decrease in the liver mitochondrial oxidation rate, as it does when given during later periods of life. One reason for this may be that the period of treatment is shorter with newborn rats than with adults, since the offspring did not survive for more than five days after parturition. An observation by CHESLER *et al.* (1942) that foetuses and offspring survive higher ethanol concentrations than do adult rats may also be of importance in this connection.

Summary

Female rats were given a 15% ethanol solution as the only drinking fluid for a fortnight before and during the whole gestation period and the effect of this treatment on their offspring was studied. The birth weight and growth as well as the respiration and phosphorylation in mitochondria from liver and brain of the offspring, were investigated.

The young of ethanol-treated females have a lower birth weight than the corresponding controls, a fact which can be explained by a difference in litter size. The difference in litter size between the two groups is, however, not statistically significant.

Ethanol-treated young had a very high mortality and lost weight during the whole period after parturition. The control young grew normally. Ethanol-treated young suckled by control females grew as rapidly as the control young.

No significant effect of ethanol treatment could be found in the liver and brain mitochondria, either in their capacity to oxidize pyruvate, glutamate and β-hydroxybutyrate or in their oxidative phosphorylation.

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Thus ethanol continuously supplied to rat foetuses during prenatal life does not cause a decrease in the liver mitochondrial oxidation rate, as it does when given during later periods of life. One reason for this may be that the period of treatment is shorter with newborn rats than with adults, since the offspring did not survive for more than five days after parturition. An observation by CHESLER *et al* (1942) that foetuses and offspring survive higher ethanol concentrations than do adult rats may also be of importance in this connection.

Summary

Female rats were given a 15 / ethanol solution as the only drinking fluid for a fortnight before and during the whole gestation period and the effect of this treatment on their offspring was studied. The birth weight and growth as well as the respiration and phosphorylation in mitochondria from liver and brain of the offspring, were investigated.

The young of ethanol treated females have a lower birth weight than the corresponding controls, a fact which can be explained by a difference in litter size. The difference in litter size between the two groups is, however not statistically significant.

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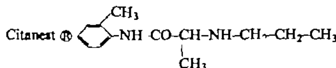
Local Anaesthetic Properties of the Optically Active Isomers of Prilocaine (Citanest®)

By

B. Åkerman, H Persson*) and C. Tegnér†

(Received September 9 1966)

The local anaesthetic prilocaine (= citanest ® = α propylamino-2 methylpropionanilide) synthesized by LÖFGREN & TEGNÉR (1960) and evaluated pharmacologically by WIEDLING (1960) and ÅSTRÖM & PERSSON (1961) can exist in two optically active forms since the prilocaine molecule (see below) contains an asymmetric carbon atom in the α position.



The resolution of the racemate into its two optically active isomers and the determination of their absolute configuration has been described by TEGNÉR & WILLMAN (1961) In the present study the local anaesthetic effects of the two isomers in animals are described The isomers were found to differ in potency in vivo but not in vitro The results are discussed against the background of what is known from other investigations on optically active isomers of compounds possessing local anaesthetic properties (e.g SCHAUMANN 1953 MOTOVILOV 1961) and with particular regard to the factors which may be related to the differences in effect, in vivo

Material and Methods

Wheal test in guinea-pigs

The compounds were injected intracutaneously on the shaven backs of guinea-pigs (300–350 g) I 0.25 ml of a 0.9 % NaCl solution at concentrations of 0 0625 to 1.0 % (pH 6.5). Each concentration was tested in 6 animals. One isomer was injected in the

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anterior area and the other isomer in the posterior area in half of the animals. This order was reversed in the other animals. The sensitivity to 6 pin-pricks within each wheal was tested every 5 min. during a 30 min. period. The number of pricks not eliciting a response during this period was added and the sum, out of 36 possible, gave an indication of the degree of anaesthesia. The method was originally described by BÜLBRING & WAJDA (1945).

Sciatic nerve block in rats and guinea-pig

0.2 ml of 0.5 1.0 and 2.0 % solutions of the isomers were injected at mid-thigh level in female rats (Sprague-Dawley 150–200 g). Half of the number of the animals were injected in one leg with one isomer After recovery the other isomer was injected into the contralateral leg. 10 animals were used for each concentration. The time f onset and time of recovery from motor paralysis were recorded. Guinea-pigs (250–300 g) were used in a second but smaller series (6 animals per concentration and isomer). The compounds were used as hydrochlorides (pH 6.9). The technique was the same as that described by SHACKELL (1935) for guinea-pigs.

Surface anaesthetic activity rabbit cornea

0.25 ml of the naesthetic solutions in concentrations from 0.25 to 2.0 % (hydrochlorides) was applied to the conjunctival sac for 30 sec. The isomers were compared on the same animals. One isomer was tested on ne eye, the other on the contralateral one, the solutions being alternated between the right and left eyes. Each concentration was tested in 6 rabbits. The reaction to stimuli from a graphite point was observed nd the time of the onset and duration of local anaesthesia measured

Blocking effect on conduction in isolated nerve

The blocking effect of the ptically active isomers of prilocaine n the nervous conduction was studied on frog (Rana pipiens) sciatic nerves, using the method of ÅSTRÖM & PERSSON (1961), with a slight modification f the technique described by MAURO TRUANT & MAC CAWLEY (1948) and TRUANT (1957). A 1.5 cm portion of the nerve between the proximal stimulation and distal recording electrodes was immersed at 20° in 4 ml bath containing the compounds. Supramaximal stimulation was performed. The duration of the monophasic pulses was 0.05 msec. The stimulatio frequency was 30/sec. and the voltage 2.5 V The effects of 1 5 2.5 5.0, 10.0 and 20 mM solutions of each isomer were studied by f llowing the reduction f the action potential for 3 or 5 min. while the nerve segment was bathed. The recovery from block was studied duri g repeated washing out of the solution. Each concentration of the two isomers was compared on one and the same nerve. Each solution was tested t least twice, the order f the experiments being randomized. The preparation was given at least 30 min. rest between each exposure to the solutions. The compounds were used as hydrochlorides and dissolved in Tasaki Ringer solution (NaCl 111.2 mM KCl 1 88 mM $CaCl_2$ 1 08 mM $NaHCO_3$ 2.38 mM and NaH_2PO 0.06 mM). This solution was also used as rinsing fluid in the experiments. The pH of all solutions was adjusted to 7.40 with 0 1 N NaOH or 0.1 N HCl.

R te of absorption of C-labelled isomers

The rate of absorption was studied in rats and rabbits using ^{14}C-labelled (TEGNÉR & DOMEIJ 1962) compounds. In one series f experiments rats were injected into the apical end of the tongue with 0 05 ml of 1.0 % solutions. Groups of 10 animals for each isomer were killed at 2 and 4 min. after injection. The tongues were rapidly taken out, weighed and

homogenized in 5 ml of 5.0% TCA. The samples as well as standards were made alkaline to a pH over 10.0 with NaOH and then shaken for 20 min. after addition of 15 ml toluene. 5 ml of the toluene extract was withdrawn and added to 10 ml of scintillation fluid (PPO/POPOP). The radioactivity of the samples was measured in Packard Tri-Carb liquid scintillation spectrometer. The ratio of the radioactivity in the tissue at 2 and 4 min. to that of the standard preparation was taken as a measure of the rat of absorption.

In a *second series* 0.25 ml of 1.0% solutions of the ^{14}C-labelled isomers was applied to the conjunctival sac of the rabbit for 1.5 min. A 0.05 ml sample of the pplied solution was withdrawn at the end of the exposure time and the remaining part was discarded. The eye was rapidly washed with 0.9% NaCl and dried free from any remaining solution. One nd the same isomer was tested on both eyes. 4 min. after being applied on one eye the solution was tested again on the other eye. The rabbits were killed 8 min. after the first pplicatio and consequently 4 min. had elapsed after the second pplication. The cornea was rapidly dissected out, weighed and homogenized in 5 ml TCA. The samples were further treated as described bove. The samples from the conjunctival sac at the end of the exposure time as well as the standards were handled in the same way. Each isomer was tested in 10 animals. The solutions used (pH 7.0 hydrochlorides) contained 1 part of the ^{14}C-labelled and 2 parts of the unlabelled compound.

$^{22}NaCl$ clearance

The effects of the isomers on the distribution of sodium ions was studied in unanaesthetized rats after intramuscular injection. Groups of 6 or 12 rats (Sprague Dawley) weighing 175–225 g were used. The hydrochloride form of the isomers was dissolved in ^{22}N labelled 0.9% NaCl solution with a specific activity of 2 µC/ml, pH 6.5. One hind limb of the rat was injected with 0.2 ml of the ^{22}Na labelled solution of one isomer and the other leg with the same volume of $^{22}NaCl$ solution alone or the $^{22}NaCl$ solution of the other isomer. The legs were injected in rapid succession. 15 min. after the injection the rats were anaesthetized with ether and bled to death. After removal of the fur the hind limbs were separated from the body and desintegrated in 10 ml fuming HNO_3 during gentle heati g. After dilution with distilled water to final volume of 50 ml, a 5 ml sample was withdrawn for determination of the radioactivity

Results

The local anaesthetic activity of the two optically active isomers of prilocaine was different in the intracutaneous wheal test in guinea pigs. The L-(+) form was more active than the D-(−) form (fig. 1a) The same difference in efficiency between the two optically active isomers of prilocaine was obtained on the rabbit cornea (fig. 1b). The L-(+) form produced surface anaesthesia in lower concentrations than the D-(−) form and at increasing concentrations with a more rapid onset (e.g. 0.5% solutions L-(+) = 1.0 ± 0.1 min. versus D-(−) = 2.5 ± 0.1 min.) and of a longer duration compared with the D-(−) form. The L-(+) form was also more effective than the D-(−) form in producing sciatic nerve block both in rats and guinea-pigs. Table 1 shows that the latent period was shorter and the duration of anaesthesia was longer with the L-(+) form

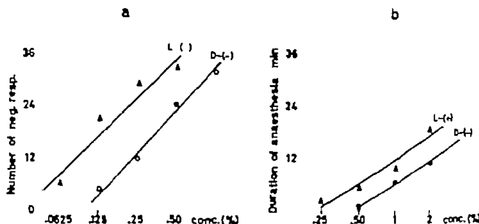


Fig. 1a. I tracutaneous wheals in guinea pigs. Effects of 0.25 ml of the isomers of prilocaine in various concentrations accordi g to the technique of BÜLBRING & WAJDA (1945). Each point represents the mean of 36 pin-pricks within 6 wheals.

Fig. 1b. Rabbit cornea. Surface anaesthetic activity of the optically active isomers f prilocaine. Volume pplied 0.5 ml Exposure time 30 seco ds.

Table 1

Sciatic nerve block in rats. Duration of local anaesthesi with different concentrations of the optically active isomers of prilocaine. Injected volume 0.2 ml. 10 animals per concentration f each isomer

Compound	Conc. %	Onset time min. m ± s.e.	Duration f motor paralysis min., m ± s.e.	Significance of difference*)	Frequency
-(+)	0.5	2.6 ± 0.3	36.7 ± 2.9	0.01 > p > 0 001	9/10
D-(−)		2.8 ± 0.2	25 9 ± 2.0		9/10
L-(+)	1 0	1 9 ± 0.1	62.8 ± 3.2	0.01 > p > 0.001	10/10
D-(−)		2 6 ± 0.2	44 5 ± 3 7		10/10
L-(+)	2.0	1 5 ± 0.2	101 0 ± 8.3	p > 0.05	10/10
D-(−)		2.9 ± 0 1	82.4 ± 7 7		10/10

) Table 1–4 Student t-test (SNEDECOR 1956).

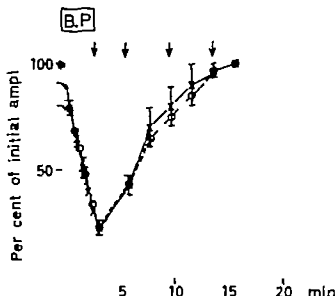


Fig. 2. Isolated frog sciatic nerve preparation. Blocking effect of the two optically active isomers of prilocaine on nervous conduction. Both compounds tested in 10 mM solutions, *pH* 7.40. *Five experiments performed with the same nerve alternating between the* L-(+) form and the D-(−) form. ×——× = L-(+) form (mean of 3 determinations). ○——○ = D-(−) form (mean of 2 determinations). ↓ = washing. B.P. = blocking period.

compared with the D-(−) form. On the other hand, on the isolated nerve preparation, the two antipodes blocked the nervous conduction to the same extent in all concentrations tested (1.25–20.0 mM). Fig. 2 illustrates the result of a typical experiment.

The finding that the local anaesthetic effect of the two isomers differed *in vivo* but not *in vitro* could hypothetically be explained by differences in the localizing properties of the two agents *in vivo*. Evidence for a slower rate of absorption of the L-(+) form as compared to the D-(−) form was obtained in the rat tongue experiments with ^{14}C labelled compounds. *Shortly after injection the amount of* L-(+) *form found in the tissue* was larger than the amount of the D-(−) form (Table 2). The results from the experiments in which ^{14}C labelled compounds were applied to the rabbit cornea show that the L-(+) form disappeared more slowly from the tissue than the D-(−) form (table 3). When the relative tissue content was estimated 4 min. after application the number of cpm/g cornea was the same for the two isomers. However, at 8 min. significantly more of the L-(+) isomer remained in the tissue than of the other form. No significant difference was observed in the rate of diffusion of the two antipodes. 92.1 ± 1.7 % of the L-(+) form remained in the conjunctival sac at the

Table 2

Rat tongue. Amount optically active isomers of prilocaine in the tissue at 2 and 4 min. after i.m. administration. Each figure represents the mean value (per cent of injected dose) from 8 animals injected with 0.05 ml of 1.0% solutions of ^{14}C-labelled compounds.

Compound	Amount in per cent of i jected dose, m ± s.e.			
	2 min.	Significance of difference	4 min.	Significance of difference
L-(+)	61.3 ± 3.0	p < 0.001	44 7 ± 3.5	p > 0 05
D-(−)	41 7 ± 2.0		38.7 ± 2.3	

Table 3

Rabbit cornea. Relative amount of optically active isomers of prilocaine in the cornea 4 and 8 min. after exposure for 1.5 min to 0.25 ml of 1.0% solutions of ^{14}C-labelled compounds.
10 animals used for each determination.

Compound	cpm/g cornea, m ± s.e.			
	4 min.	Significance f difference	8 min.	Significance of difference
L-(+)	13455 ± 1107	p > 0.05	13077 ± 1612	0.01 > p > 0.001
D-(−)	13746 ± 2224		7822 ± 1016	

end of the exposure time as against 86 0 ± 3 4 / for the D-(−) form. Table 4 shows that both isomers delayed the clearance of ^{22}Na from the rat thigh, the L-(+) form being more active than the D-(−) form in this respect.

Discussion

The effects of optically active isomers of compounds possessing local anaesthetic properties have been reported previously AF EKENSTAM, EGNÉR & PETTERSSON (1957) obtained no difference in local anaesthetic potency between the isomers of n-methylpipecolinoyl 2,6-xylidine. The effects of the optical isomers of diethylamino-2,6-dimethylpropionilanilide were also similar (KUDRYASHOVA & KHROMOV BORISOV 1960 MOTOVILOV 1961) while the (−) isomer of diethylamino-2,4-dimethyl-

Table 4

^{22}Na clearance in rats. Effect of 0.2 ml of the optically active isomers of prilocaine on the ^{22}Na clearance 15 minutes after i.m. injection (1.0 % solutions). The effect expressed as the difference between the two isomers in mount of ^{22}N recovered in per cent of injected dose.
5 animals used for each solution.

Compound	Difference m ± s.e.	Significance of difference
L-(+) 0.9 % N Cl	21 4±2.5	$p<0.001$
D-(−) 0.9 % NaCl	12.1±1.9	$p<0.001$
L-(+) D-(−)	9.5±2.0	$0.01>p>0.001$

propionil anilide was more active than its optical antipode, in tests for surface and conduction anaesthesia.

The present study has demonstrated that the L-(+) form of prilocaine (α-propylamino-2-methylpropionanilide) is more active than the D-(−) form in wheal and nerve block experiments and on surface application. The effects of the two compounds *in vitro* on the impulse propagation in the isolated nerve were, however of the same order

The difference in effect between the isomers of prilocaine *in vivo* cannot be explained by a difference in metabolic breakdown rate as suggested by SCHAUMANN (1953) for isomers of cocaine. Prilocaine is not, like cocaine, hydrolyzed in the plasma (GEDDES 1965 ÅKERMAN *et al* 1966) and although the D-(−) form is more rapidly broken down than the L-(+) form (ÅKERMAN unpublished results), this occurring mainly in the liver it seems less probable that this difference in rate of metabolism would for instance explain the marked differences in local anaesthetic action on the rabbit cornea. It is also possible that there is a difference in the rate of penetration of the two isomers to the site of action in the axon membrane. This explanation does, however seem to be contradicted by the findings that the rate of diffusion into the rabbit cornea and the blocking potency on the isolated nerve was the same for the two antipodes. The latter result also seems to exclude the possibility that as far as the isomers of prilocaine are concerned, the steric factors would influence the mechanisms which produce a block of the nervous conduction. It does not, however seem improbable that steric factors are able to exert an effect at the site

of action for local anaesthesia. Experiments with other pairs of optically active isomers of compounds having local anaesthetic activity have shown that differences in potency between isomers are also present in isolated nerves, suggesting a stereospecific receptor mechanism for local anaesthetic effect (ÅKERMAN unpublished results) Since no difference in effect between the isomers of prilocaine could be demonstrated *in vitro* it is probable that the intrinsic difference at the site of action is either absent or very small in the case of this pair of isomers.

The most probable explanation for the more marked effect of the L-(+) form *in vivo* as compared with the D-(−) form, is that the former compound is better localized at the site of application Thus suggestion is based on the findings that the D-(−) form disappeared more rapidly than the L-(+) form both from the rat tongue and the rabbit cornea. The fact that the L-(+) form delayed the clearance of ^{22}Na to a greater extent than the D-(−) form indicates that the effects of the two optically active isomers of prilocaine on the local vascular bed were different, since the clearance of radioactive sodium ions to some extent is dependent on the local circulation (KETY 1949) The more marked local anaesthetic effect of the L-(+) form as compared to the D-(−) form may thus be related to better binding properties and/or to some different effect on the peripheral vascular bed.

Summary

The local anaesthetic action of the optically active isomers of α-propylamino-2 methylpropionanilide (prilocaine citanest ®) was studied in animals. The L-(+) form was more active than the D-(−) form in wheal tests, at nerve block and on surface application. The blocking effect of the nervous conduction in an isolated nerve was, however the same for the two isomers Evidence has been produced that the L-(+) form is more slowly absorbed than the D-(−) form. The L-(+) form delayed the clearance of radioactive sodium ions to a greater extent than the D-(−) form.

It is suggested that the more marked local anaesthetic effect of the L-(+) form *in vivo* as compared with that of the D-(−) form depends on a better localization at the site of application of the former compound, which in turn may be related to more favourable binding properties and/or to some different action on the local vascular bed. It is less likely that the different rates of metabolism of the two isomers may account for the differences in the local anaesthetic action *in vivo* The possibility that in general steric factors may exert an effect at the site of action for local anaesthesia is also discussed

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A Method for Porta-Cava Anastomosis in the Rat*

By

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The object of this paper is to describe an operative procedure by which it is possible, in rats, to exclude totally the liver from the circulation, and at the same time keep the circulation and functions of the remaining abdominal organs intact.

Method

The principle is that the liver is excluded totally from the circulation by ligating both the common hepatic artery and the inferior vena cava proximally and distally to the liver round polyethylene tube, which has previously been introduced into the inferior vena cava. The blood from the portal vein is led through a cannula provided with a silicon rubber tube into an open container from where it is led directly into the cava-catheter by means of pump.

Albino female rats of the Wistar strain, weighing 200–300 g are used for the operation. A polyethylene tube, in which two thirds of the circumference is removed from 2.2 cm piece of the middle of the tube is used to canalize the inferior vena cava. The internal diameter of the tube is 1 mm and the thickness of the wall is 0.25 mm. Cannula No. 12 and silk ligatures No 1 are used Finally ordinary surgical instruments (fig. 1) and peristaltic pump (Heidolph Schwabach) with capacity of 1–5 ml/min are used.

Without any preoperative anticoagulation treatment the animals are anaesthetized with mebumal-sodium (nembutal ®) 80 mg/kg intraperitoneally placed on a heated surgical table (30°) and a tracheotomy is performed, after which metal cannula is inserted into the trachea. The respiration is controlled mechanically with respiratory volume of 4–6 ml O_2/min. and frequency of 49 per minute.

A polyethylene tube containing heparin saline is inserted into the left external carotid artery This tube is connected via transducer to recorder for continuous registration of the arterial blood pressure.

* A preliminary report of this investigation was given at the XII Scandinavian Congress of Physiology Turku, Finland, 1966.



Fig. 1 Surgical instruments used for the porta-cava shunt operation. The polyethylene tube used for the cava catheter is shown. Two thirds of the circumference in the middle 2.2 cm of the tube has been removed. The metal thread used as an introducer is seen.

The bdomen is pened in the linea alba with an incision, which is extended laterally to the right over the iliac vessels. The falciform ligament is incised along the upper side of the liver as far as the inferior vena cava, round which loose ligature is placed. Another loose ligature is placed round the inferior vena cava below the liver just proximally to the inlet of the renal veins. About 1 cm of the right common iliac vein just distally to the bifurcature, is exposed and two ligatures are placed, of which the distal one is tied. The vein is opened and the cava-catheter inserted by means of thin metal thread, which serve as ntroducer The point of the cava-catheter is placed just below the right trium. How much of the catheter has to be introduced into the common iliac vei in order to be placed properly is estimated externally the animal before each operation. At the same time the position of the excision in the cava-catheter is controled. The upper part of the excision should be on level with the nlet of the renal veins. Fig. 2 shows the result of the operation after introduction of the cava-catheter int the common iliac vein and inferior vena cava. The placing of the excision in relation to the renal veins the lumbar and iliolumbar veins as well as to the cava ligature below the liver is also shown. The proximal ligature on the common iliac vein is tied round the cava-catheter The common hepatic artery is ligated in front of the portal vein between the latter and the bile duct. In porta hepatis the portal vein is ligated just before the branching of the vein. Another ligature is placed loosely around the portal vein just proximally to the superior mesenteric vein. A 2 cm long cannula provided with silicon rubber tube is introduced into the vein, and is fastened by the distal ligature (fig. 2). The silicon rubber tube runs freely into an open container holding about 8 ml of heparinized blood from donor rat. Another silicon rubber tube leads from this container via peristaltic pump to the cava-catheter The pump is started simultaneously with the tying-up f the first ligature round the portal vein. The ligatures on the inferior vena cava under and over the liver are tied in the order mentioned and the liver is left in situ. The bdominal contents are carefully covered with plastic foil during the operation. The operation takes about half an hour

The pumping into the inferior vena cava is effected at speed of 1.5 ml/min. At regular

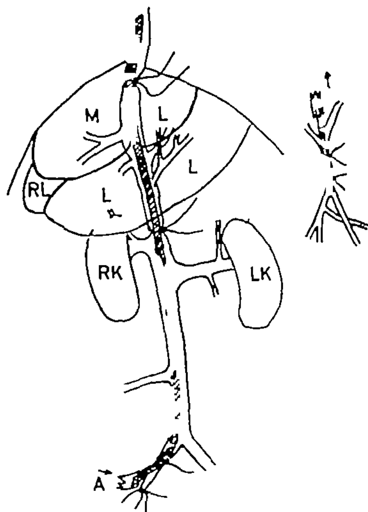


Fig. 2. A sketch of the principle of the operation. The position of the polyethylene catheter in the cava inf. is shown. The tube has diameter of 1 mm and its wall is 0.25 mm in thickness. Two thirds of the circumference in the middle 2.2 cm of the tube has been excised in order to facilitate the blood flow from the vv. renales and vv. iliolumbales. The ligatures proximal and distal to the liver are shown. On the right of the sketch the cannulated v. portae is seen. The point of the cannula is placed just proximal to the v. mesenterica sup. Blood from the portal vein runs passively into an open container with heparinized donor blood. The blood from the container is pumped into the cava catheter at A.

intervals the outlet from the portal vein is measured. The liver function is examined by means of bromsulphthalein, 6 mg/kg being injected into the left external carotid artery. Blood is sampled 6 and 40 minutes after the injection for the determination of the bromsulphthalein content in the plasma (ROSENTHAL & WHITE 1925). Liver function tests with bromsulphthalein were performed o a rat in which the operation had been completed, except for the tying of the ligatures round the inferior vena cava and the common hepatic

Table 1

Liver function tests in 3 rats after administration of bromsulphtalein 6 mg/kg i.a. In the first case the porta-cava anastomosis is done as described. In the second case the ligatures round the cava inf and the a. hepatica com. are loose. In the last case only the external carotid artery is cannulated in an anesthetized rat. The initial concentration of bromsulphtalein is measured when bromsulphtalein is considered to be distributed in the total plasma volume (2–3 minutes after administration).

	Per cent of initial value after	
	25 min.	40 min.
Without hepatic circulation		100
After sham operation	35	
Control	0	

artery. In another anesthetized rat, catheter was inserted into the left external carotid artery from which blood was sampled to measure the concentration of bromsulphtalein and of potassium in the plasma. The concentration of potassium in the plasma was finally determined in two animals with established porta-cava anastomosis.

Results

The figures in table 1 show that rats, with intact liver circulation, *eliminate* 100 % of the bromsulphthalein administered in the course of 25 minutes. 65 % of the bromsulphthalein administered is *eliminated* in the course of 25 minutes in animals in which porta-cava anastomosis has been performed, but with intact liver circulation. In rats, with complete operation, i.e. all ligatures are tied, 100 % of the bromsulphthalein administered is *recovered* during the testing period (40 minutes).

Table 2

Determinations in two rats of the concentration of potassium in the plasma 2½–4½ hours after the operation.

Min. after oper	meq. K/l	Rat no.
150	3.1	I
220	2.7	II
250	2.9	II
255	3.1	II



Fig. 3 A recording of the arterial blood pressure 30, 60, 120 and 240 minutes after the terminatio of the operation. Abscissa minutes. Ordinate mm Hg.

Figure 3 shows a section of the arterial blood pressure 30, 60, 120 and 240 min. (4 h.) after the operation. The systolic blood pressure varies between 50 and 60 mm Hg.

The plasma potassium values vary from 2.7 to 3.1 meq/L (table 2).

The outflow from the portal vein was 1–2 ml/min. throughout the testing period. The survival time was 2–4 hours in 10 operations, and the systolic blood pressure varied between 40 and 70 mm Hg.

Discussion

Anastomoses between the portal and caval veins have previously been performed on experimental animals, among others on cats (JOOP MADSEN 1960). However principles previously reported cannot be adopted to the rat, because of anatomical characteristics. LEE & FISHER (1961) performed a porta-cava anastomosis on rats, but did not give any quantitative data about the extent to which the liver function had ceased. That the function of the liver had not ceased completely is evident from the

fact that the animals survived for 7 months. In the present work the liver is totally excluded from the circulation as indicated by the figures in table 1

The dimensions of the catheter used are of crucial importance for a successful result. The quantity of liquid per time unit, capable of passing through a given tube is, according to the law of Poiseulle on laminar flow dependent on the length of the tube. The longer it is, the higher the pressure gradient has to be, to get the same quantity of liquid through the tube. In a series of initial experiments the blood was led from the portal vein through a short silicon rubber tube directly into the inferior vena cava catheter The silicon rubber tube had a larger diameter than the cava catheter so as to further diminish the resistance in the tube system. However the blood pressure continued to decrease, indicating that there was too great a resistance in this system. We therefore introduced a peristaltic pump in the extracorporal circulation between the portal and the vena cava by which, as shown in figure 3 it is possible to keep the blood pressure between 50–60 mm Hg. The low blood pressure can be raised by an increased pumping into the cava catheter but the passive outlet from the portal vein did not at any time exceed 2 ml/min. The pump was therefore adjusted to a flow of 1 5 ml/min. The anaesthesia used (mebumal-sodium) may possibly account for the low blood pressure.

The excision in the cava-catheter described, apart from diminishing the length of the tube, is of value in draining the blood from the abdominal veins. No dilatation of either the renal veins or the inferior vena cava after the trying-up of the ligature under the liver was seen. Further more, urine was found in the bladder at post mortem, and in a few determinations on the content of potassium in the plasma, normal conditions were found (table 2).

A series of other factors are of significance for a succesful surgical result. Primarily the inferior vena cava should not be freely dissected as the connective tissue sheath round the vein contributes to the maintenance of the negative pressure in the vein and thereby the central blood flow Secondly it is necessary to do a free dissection of the common iliac vein carefully since the catheter will otherwise be caught in connective tissue pockets and form a false canal. Finally evaporation and cooling should be prevented as far as possible during the whole operation.

Summary

A description is given of a surgical method for a porta-cava anastomosis, by which it is possible in rats to exclude the liver totally from the circulation, without interfering with the circulation of other abdominal organs

or of the musculature. The inferior vena cava is ligated proximally and distally to the liver round a polyethylene tube, where two thirds of the circumference in the central 2 cm of the tube has been removed in such a way that the outlet from the abdominal veins can take place normally. The outflow from the portal vein takes place passively through a cannula introduced into the vein, down into an open container with heparinized blood from a donor rat. From the container the blood is pumped by means of a peristaltic pump into the cava-catheter. The time used for the surgical procedures is half an hour and the liver is left in situ. The postoperative survival time is 2–4 hours, and the blood pressure at the end of the operation is 50–60 mm Hg. Liver function tests with bromsulphthalein show postoperatively that the liver is completely excluded from the circulation. Plasma potassium values postoperatively were normal.

Acknowledgements

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The bromsulphthalein was generously supplied by The University Hospital Pharmacy, Copenhagen, (head: Professor Svend Aage Schou, Ph.D.)

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From the Section of Forensic Chemistry (Fanny Halstrøm, Ph.D.) The Department of Pharmacology University of Copenhagen (Professor Knud O Møller M.D.)

Interfering Substances in the Determination of Poisons in Autopsy Material Nicotinamide, Uracil and Thymine

By

Bent Kæmpe

(Received August 20, 1966)

In the spectrophotometric analysis of extracts of fresh autopsy material (liver and gastric contents) *for the content of alkaloids within the ultra* violet range distinct absorption bands are often seen which can be attributed to three or four substances extracted at the same time. We have identified three of these substances as nicotinamide, uracil, and thymine.

The methods of isolation and identification of the substances are described below together with their analytical properties.

Methods

General technique

Physico-chemical constants were determined as described previously (Kæmpe 1964). The spectra within the infrared range were recorded with Beckman spectrophotometer model I.R. 10 and KBr tablets.

A) *Nicotinamide*

For analysis single human liver stored at $-18°$ *for one month was used. The liver was* removed at autopsy from woman, aged 30, who was believed to have died of encephalitis. The forensic-chemical analyses revealed no poisons. The liver showed no signs of petrefac tion.

A extract of the liver was prepared by procedure commonly used in this country for the isolation of alkaloids and similar substances.

To 50 g of homogenised liver tissue was added 30 g $NaHCO_3$ and 2 N-NaOH to pH of bout 9 Extraction was then performed in separating funnel with 150 ml of chloroform-isopropanol mixture (3 + 1 /v). Mechanical shaking was performed for 20 minutes with total of 450 ml of the extracting gent. T the combined, dehydrated, organic phases two

drops of 8 N HCl were added, after which the extracting agents were evaporated on a boiling water bath. For purification the residue was dissolved in 20 ml of hot 0.1 N-H_2SO_4, then cooled and filtered. This procedure was repeated three times with total of 80 ml of 0.1 N H_2SO_4. The combined filtrates were extracted once with an equal volume of ether, which was discarded. To the aqueous phase 8 N NaOH was added to produce strong alkaline reaction (pH about 13). Then the product was shaken three times, each time with an equal volume of chloroform (*b* containing pure bases). Finally 8 N H_2SO_4 was added to bring about an acid reaction and then $NaHCO_3$ in excess (pH = 8.2). Extraction was then performed three times each time with an equal volume of chloroform-isopropanol (3 + 1) which contained bases of an amphoteric character (morphine and related substances). The dehydrated, organic phases were evaporated and the residues *b* and *c* were transferred with a mixture of chloroform-metanol (1 + 1 /v) to Whatman paper no 3 pH 7.0. The ascending chromatogram was developed with a mixture of di-n-butyl-ether water and amylene hydrate 7:13 80 (BROOM *et al.* 1955). Observation of the chromatogram in short-wave light (254 mμ) revealed a dark spot with an R value of 0.67 on the band with the residue *b* Along the band containing residue *c* three dark spots having R_F values of 0.4.., 0.60, and 0.67 were found The areas corresponding to the spots with R_F value 0.67 were cut out and eluted with 0.2 N HCl. 4–5 ml was collected from each spot. Measuring at acid and alkaline reactions in ultraviolet light, showed the same conditions of absorption for the eluates as are obtained by measurement of nicotinamide (see Fig. 2). The eluates from *b* and *c*, estimated to contain 0.3 mg of a substance believed to be nicotinamide were mixed and, after addition of $NaHCO_3$ in excess, extracted four times, each time with 50 ml of chloroform-isopropanol. The combined, dehydrated, organic phases were evaporated to dryness. The residue was sublimated at a temperature of 100° and pressure of 5 mm Hg.

B. *Uracil*

The liver used for the extraction of this substance was removed at autopsy from a man aged 60. Death was due to poisoning by ethanol and barbiturates. The liver had been stored at 4–5° for 14 days and at −18° for 2 months. It showed n signs of putrefaction. The procedure was the same as described under A with regard t extraction of the liver tissue and purification of the extract dissolved in 0.1 N H_2SO_4. T the combined, cooled, and filtered 0.1 N H_2SO_4 solutions $NaHCO_3$ was added in excess (pH = 8.2), after which extraction was performed three times (*c*), each time with double volume of chloroform-isopropanol. *c* was purified by paper chromatography as described under A. Observation in 254 mμ light revealed three dark spots with the same R_F values as stated under A. The area corresponding to the spot with the R value 0.42 was cut out and eluted with 0.2 N HCl. Measurement of the eluate (8 ml) at acid and alkaline reactions in ultraviolet light showed the same conditions of absorption as are obtained by measuring about 0.4 mg uracil (see Fig. 4). The eluate was extracted and sublimated at 165° as described under A.

C. *Thymine*

The liver was removed at autopsy from a man, aged 21 who had been in poor health and was believed to have died from influenza. The forensic-chemical analysis disclosed no poisons, except traces f sulphametoxypyridazine. The liver had been stored in a minced state at 4–5° for one month. An extract was prepared, which was purified as described under B. 10 ml of the luate collected from the area corresponding to the spot with R value 0.60 gave similar spectrophotometric curves as were obtained by measuring 0.2 mg thymine (see Fig. 6). The eluate was extracted nd sublimated as described under B.

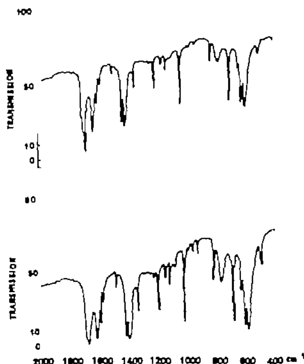


Fig. 1 Infrared spectra of the isolated substance (the upper curve) and nicotinamide (the lower curve) in KBr

Results

The sublimate isolated by extraction A showed a m.p of 116 (corr). Its spectrum was recorded within the range of 2000–400 cm⁻¹ see Fig. 1 As material for comparison we used a purchased sample of *nicotinamide* Ph.D This sample had a m.p of 116 Its spectra within the ultraviolet and infrared ranges were recorded, see Figs. 2 and 1 Its R_F value on Whatman no 3 pH 7 developed according to BROSSI *et al.* (1955) system A, was 0.68 The mixed melting point of a mixture of the substance isolated from the liver and nicotinamide was 116

The infrared spectrum of the sublimate isolated by extraction B is seen in Fig. 3 As material for comparison a purchased specimen of *uracil* Sigma ® was used. The spectra of this specimen within the infrared and ultraviolet ranges were recorded (see Figs. 3 and 4). Its R_F value in the stated chromatographic system was found to be 0.43

The sublimate obtained by extraction C gave the curve shown in Fig. 5 within the infrared range. The spectra within the infrared and ultraviolet

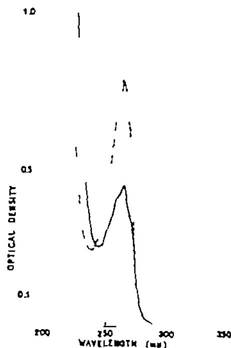


Fig. 2. Ultraviolet spectra of nicotinamide (about 18.2 μg/ml) in 0.2 N-HCl ---- and in 0.1 N-NaOH ——

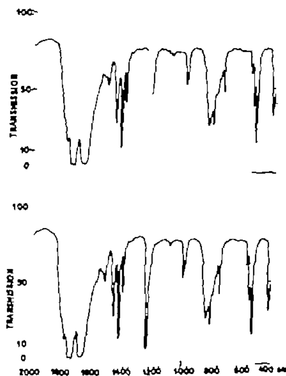


Fig. 3 Infrared spectra of the isolated substance (the upper curve) and uracil (the lower curve) in KBr.

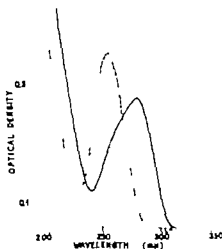


Fig. 4 Ultraviolet spectra of uracil (bout 9.1 µg/ml) in 0.2 N HCl – – – and in 0.1 N NaOH ——

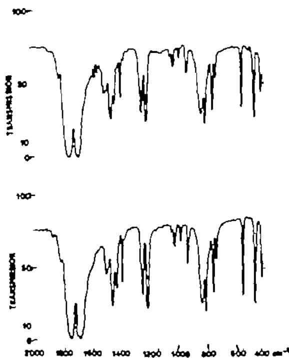


Fig. 5. Infrared spectra of the isolated substance (the upper curve) and thymine (the lower curve) in KBr

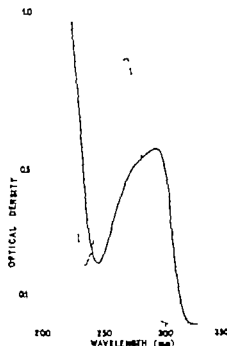


Fig. 6. Ultraviolet spectra of thymine (about 13.6 µg/ml) in 0.2 N HCl ---- and in 0.1 N NaOH ——

ranges of a purchased specimen of *thymine* Sigma ® were recorded (Figs. 5 and 6) Its R_F value was found to be 0 61

The values obtained for the substances A, B and C isolated from the liver were found by comparison to be identical with those for nicotinamide, uracil, and thymine respectively

The three isolated substances were as stated, found to be present in relatively high concentrations in extracts from liver tissue analysed for alkaloids, and displayed distinct absorption bands within the ultraviolet range. Mention will therefore be made below of their extraction, R_F values and reactions to commonly used alkaloid reagents.

Extraction, R_F values and reactions to commonly used alkaloid reagents for nicotinamide uracil, and thymine

Distribution coefficients for nicotinamide, uracil, and thymine are shown in Table 1

R_F values for nicotinamide, uracil, and thymine as well as their reactions to alkaloid reagents are shown in Table 2. R_F values for a few commonly occurring alkaloids are shown for comparison

Table 1

Distribution coefficients at room temperature of nicotinamide, uracil, and thymine, in

A 0.1 N NaOH with excess $NaHCO_3$ added (pH 9.0)/chloroform-isopropanol (3 + 1 v/v)
B 0.1 N NaOH/chloroform, and
C: 0.1 N NaOH/ether

Substance	A	B	C
Nicotinamide	1.8	15.8	15.8
Uracil	7.9	almost ∞	almost ∞
Thymine	3.5	almost ∞	almost ∞

It is seen in Table 1 that nicotinamide, unlike uracil and thymine, is to some extent extracted as a "*b* substance" (e.g. nicotine). As, it has, moreover, a nicotine-like absorption curve in ultraviolet light, its presence will interfere with the spectrophotometric determination of nicotine, unless the two substances have been separated before the measurement. Separation can be undertaken by chromatography as shown in Table 2, in which it is also seen that the precipitate of nicotinamide differs in colour from that of nicotine. It is also worth noting that uracil and thymine do not form a coloured precipitates with the alkaloid reagent, iodoplatinate.

Discussion

Metabolites of poisons must be included in the forensic-chemical analysis, to allow the estimation of the amount of poison ingested. Metabolites of poisons are generally more water-soluble than the original poison. For the extraction of poisons and their metabolites we should therefore, as far as possible, employ extracting agents such as chloroform-isopropanol (3 + 1) and saturate the tissue extract with an inorganic salt (e.g. $NaHCO_3$). As metabolites of alkaloids may be of an amphoteric character (introduction of an OH-group), the extraction should be performed at a pH value close to 8.4 (excess of $NaHCO_3$), the distribution coefficient of amphoteric substances in chloroform-isopropanol (3 + 1)/water being highest at their iso-electric point. As is well-known, nicotinamide, uracil and thymine form part of many rather common nucleotides and become liberated from these under the influence of an acid. These substances must therefore be expected to be present in nearly every chloroform-isopropanol extract, when gastric contents and fresh liver

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Oedema Formation in Mice Induced by Percutaneous Iontophoresis with Histamine and 5-Hydroxytryptamine

By

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(Received October 11 1966)

In a previous publication from this laboratory the progress and composition of the oedema fluid induced in mice by the application of xylene on the skin was described (LANGGÅRD HVIDBERG & SZPORNY 1964). The object of the present work was to elucidate the role played by the local liberation of histamine and serotonin (5-hydroxytryptamine) in the development of such an inflammatory reaction caused by chemical irritation. The effect of the two biogenic amines was examined after their introduction into the loose interstitial connective tissue of the corium and the subcutaneous tissue by means of iontophoresis.

Method

Male, albino mice, 5–6 weeks old, of a single strain (Leo, Strain) were used. While the animals were under halothane (fluothane ®) anaesthesia, two 3.5 cm² symmetrical, semilunar areas were marked on the depilated skin of the back (cf SECHER-HANSEN, LANGGÅRD & SCHOU 1967 a). The animals with their abdomen lying on piece of cotton wool moistened with solution of 0.9 % N Cl in water were placed on 10 × 11 cm copper sheet acting as an indifferent electrode and connected in series with a milliampere meter and the negative pole of 16 V D C. battery The different electrode shaped so as to cover one of the areas marked, was provided with a thin piece of cotton wool saturated with a freshly prepared, aqueous solution of either 2 % histamine diphosphate or 2 % serotonin creatinine sulphate. This electrode was by a sliding rheostat (2100Ω) connected in series with the milliamperemeter and the positive pole f the battery and placed bove the right side area (Fig. 1). The iontophoresis was undertaken at 4 mA for 2 minutes. In this way it is possible, according to the electrolytic laws of Faraday to induce $\frac{M\ A\ t}{96500}$ g of the elec-



Fig. 1 Halothane (fluothane ®) anaesthetized mouse, placed on copper sheet as the negative electrode. The animal has been marked symmetrically on the depilated skin of the back. The semi-lunar shaped copper plate is shown as positive or driving electrode.

trolyte in question (M = the molecular weight, A = ampere, t = time in seconds, n = the ionic valence). When the molecular weight of the histamine base and the serotonin base are 111 and 176 respectively it is possible with the values stated to induce maximally 552 µg histamine base and 880 µg serotonin base.

Five or 15 minutes, 2½, 24 or 48 hours after the end of the histamine iontophoresis, and 15 minutes, 2½ or 48 hours after the end of the serotonin iontophoresis, the animals were stunned by a blow on the neck, decapitated and bled. The two pieces of skin (including the corium and the subcutaneous tissue) were cut out and immediately weighed on torsion balance. An increase in weight due to oedema formation caused by the iontophoresis was determined as the difference in weight in mg between the two sides, cf. SECHER-HANSEN, LANGGÅRD & SCHOU (1967a). The contents of water and of fat-free solids were determined by freeze drying, followed by extraction with petroleum-ether and ether. The histamine content was determined spectrofluorometrically as condensation product of histamine and ophthalaldehyde (cf. ZACHARIAE 1965). In all, 10 mg dry fat-free tissue was used. Serotonin was determined biologically by the method of VANE (1957), modified by JANSEN (1967). Histamine and serotonin were thus expressed in relation to the fat-free solids, but were converted, by means of the values for the total of fat-free solids on the right and left sides, to the total of histamine and of serotonin on the two sides. The differences between the totals of the two sides thus constituted the quantities of histamine and serotonin induced by iontophoresis.

In one control group iontophoresis was undertaken with the same experimental procedure, using 0.9 % NaCl at 4 mA for two minutes. The animals were killed 15 minutes after the iontophoresis, and thereafter treated as described above. In another control group the total histamine and serotonin contents on the two sides were determined separately in the

corium and the subcutaneous tissue, 5 and 15 minutes as well as 2½ hours after iontophoresis with 2 % histamine diphosphate as well as with 2 / serotonin creatinine sulphate. Finally iontophoresis was undertaken, in two recently killed mice with the same experimental procedure, using 2 / histamine diphosphate.

Results

The figures in table I show that iontophoresis with 0.9 / NaCl does not produce any significant oedema on the treated side, and that there is no significant difference between the content of histamine on the two sides.

Table 1

The difference in weight and histamine content between two symmetrical pieces of skin with a surface area of 5.5 cm² from the same animal, 15 minutes after iontophoresis with 0.9 / NaCl at 4 mA for 2 minutes on the right side.

Wet Weight			Total Histamine		
Iontophorized mg		— Control Side s.e.m.	Iontophorized mg		— Control Side s.e.m.
4	±	3.2	2	±	0.6
— 18			n — 5		

The figures in table 2 show that by iontophoresis, histamine and serotonin are introduced not only into the corium but also into the subcutaneous tissue. Two and a half hours after the termination of the iontophoresis, histamine values in the subcutaneous tissue are *normal*, i.e. corresponding to those of the control side, whereas the content of serotonin is still significantly increased.

The total surplus of histamine on the treated side is shown as a function of time (abscissa = $\log_{10}$ time in minutes) in fig. 2. In the same figure, the weights of the corresponding oedemas are indicated in mg. The s.e.m. values are shown by vertical lines through the maxima. It appears that with a histamine surplus corresponding to 7 times the normal content (on an average 27 μg histamine base in 52 animals) an average oedema of 28 mg is produced in a piece of skin of this size (5.5 cm²). With smaller excess *of histamine smaller oedemas are found.* In this semi-logarithmic system the histamine content decreases more or less rectilinearly. By extrapolation to the time O corresponding to the time of termination of the iontophoresis, it is found that on an average, 217 μg histamine base has been introduced. This is in good agreement with the results from the

Table 2

The total contents of histamine base and serotonin base in a defined piece of skin with a surface area of 5.5 cm² 5 15 and 150 minutes (2½ h.) after iontophoresis with 2 % histamine diphosphate or 2 % serotonin creatinine-sulphate at 4 mA for 2 minutes. The subcutaneous tissue was separated from the corium and the layers analyzed separately

	Total Histamine				Total Serotonin			
	Histamine base µg Side with Iontophoresis		Histamine base µg Control Side		Serotonin base µg Side with Iontophoresis		Serotonin base µg Control Side	
	Corium	Subcutaneous Tissue	Corium	Subcutaneous Tissue	Corium	Subcutaneous Tissue	Corium	Subcutaneous Tissue
	60.0	29.6	20.0	6.4				
5 minutes	75.[illegible]	29.2	26.4	8.4	26.9	7.6	0.2	0.1
	79.2	40.4	21.1	6.1	30.9	21.0	0.4	0.2
	38.7	21.3	22.2	6.6				
15 minutes	56.1	26.4	27.3		15.5	5.1	0.4	0.3
	43.7	33.8	21.8	10.7	31.1	7.2	0.2	0.1
	23.5	2.5	16.0	3.2				
150 minutes	20.1	6.9	15.1	6.2	3.6	8.6	0.2	0.2
	8.4	6.2	5.6	2.1	2.5	10.0	0.2	0.1

examination of the quantity of histamine introduced under identical circumstances, but on dead mice. Here, on an average 214 µg histamine base (2 determinations) was found, i.e. about 40 % of the calculated maximum.

In the same way the differences between the total serotonin content on the right and the left side after serotonin iontophoresis and the corresponding differences in weight in mg between the two sides, are indicated in fig. 3 Fifteen minutes after the iontophoresis an excess serotonin is found on the treated side, which is about 90 times the normal content (on an average 0.4 µg in 17 animals), but this concentration is not oedema producing. Two and a half hours after the iontophoresis, the increase in serotonin is about 6 times the normal content. At the same time an average oedema of 66 mg is found. 48 hours after the iontophoresis the serotonin content on the treated side is still twice as high as that on the control side, and a small, but significant oedema is found.

Fig. 2. Freshly prepared aqueous solution of 2% histamine diphosphate was introduced by iontophoresis on the right side of anaesthetized animals. The figure shows the difference in the total content of histamine base between the treated and the untreated symmetrical (5.5 cm²) areas, i.e. the histamine base excess in μg open columns. (A control side contains 27 ± 0.6 μg histamine base (n = 52)). The difference in wet weights in mg between the treated and the untreated side is shown by crossed columns. s.e.m. -values are shown by vertical lines through the maxima. The difference in wet weight between the two side 150 minutes (2½ h) after xylon application was 230 mg

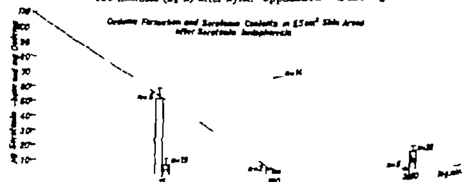


Fig 3. Freshly prepared aqueous solution of 2% serotonin creatinine sulphate was introduced by iontophoresis on the right side f anaesthetized animals. The figure shows the difference in the total content of serotonin base between the treated and the untreated symmetrical (5.5 cm²) areas, i.e. the serotonin base excess in μg open columns. (A control side contains 0.4 ± 0.03 μg serotonin base (n = 17)). The difference in wet weights in mg between the treated and the untreated side is shown by crossed columns. s.e.m. values are depicted by vertical lines through the maxima. The difference in wet weight between the two sides 150 minutes (2½ h) fter xylene application was 230 mg.

(LANGGÅRD, HVIDBERG & SKPORNY 1964).

Discussion

When xylene is applied to the skin of mice, an oedema is produced, which attains a maximum after 2½ hours. At this time the inflamed skin defined by its surface area, weighs twice as much as the normal skin (cf. LANGGÅRD HVIDBERG & SZPORNY 1964) For the development of these oedemas local liberation of biogenic amines is assumed to be of importance (surveys WILHELM 1962 ZACHARIAE 1965).

Examinations on the significance of biogenic amines in the acute inflammatory reaction have been organized on one of two main principles The inflammatory reaction was produced by unspecific stimuli and the local liberation of amines measured, or an excess of the amines introduced into the skin and the development of the inflammatory reaction followed. The former principle involves calculatory problems which are seldom considered (LANGGÅRD & SECHER HANSEN 1967). Moreover it may not be justifiable to conclude from an alteration in the content of amines in the tissues that amines affect the function during the conditions under discussion. In view of these objections, the latter of the principles mentioned is presumably to be preferred. However investigations carried out on this principle may also be subject to criticism. The amines are often introduced by injection. Such experiments are defective in that the injection *per se* represents a trauma, by which endogenous amines may be liberated (SCHOU 1961 SECHER HANSEN LANGGÅRD & SCHOU 1967b). In the present work histamine and serotonin have, therefore, been introduced into the skin by iontophoresis, so that the skin is not injured and no biogenic amines are liberated (Table 1)

The technique used for marking two symmetrical areas, implies that exact quantitative data on the amount of amine introduced at any time can be correlated to the quantitative and qualitative data on the inflammatory response (cf SECHER HANSEN, LANGGÅRD & SCHOU 1967a & b)

The results indicate that neither histamine nor serotonin play any quantitatively dominating role in the development of the inflammatory oedema in mice. In particular the histamine induced oedema is small, in agreement with the view that the white mouse is relatively resistant to histamine (KALLOS 1957 DHAR & SANYAL 1963) Iontophoresis with histamine caused an increase in weight which in a piece of skin defined by its surface area was only 10 % at its maximum. This maximum was reached after 5 minutes, which tallies with the assumption that histamine is of importance in the earliest phase of the inflammatory reaction (SPECTOR & WILLOUGHBY 1959). After iontophoresis with serotonin the weight of the skin sample treated rose maximally by 25 %. This increase

was observed 2½ hours after the iontophoresis Two possible explanations of this delayed effect may be offered Serotonin may be of importance in a later phase in the inflammatory reaction, or serotonin produces oedema only in low concentrations. The latter explanation seems to be the more probable. According to most investigators, high concentrations of serotonin, such as are found immediately after the iontophoresis, cause vasoconstriction and not vasodilatation and formation of oedema. SPECTOR & WILLOUGHBY (1957) *found that concentrations from 0.1-10* µg/ml caused vasodilatation, whereas higher concentrations brought about vasoconstriction. In the present investigation the concentration of serotonin was 14 µg/ml tissue fluid, 2½ hours after the iontophoresis. The results of WEIS (1963) who injected histamine and serotonin into mouse paws, agree fairly well with those obtained here. However it is not possible on the basis of WEIS's results either to decide whether serotonin is of greater importance to a later phase of oedema formation. For a complete account of these relations the oedema formation would have to be examined both as a function of time and as a function of the dose of biogenic amines. Because of *difficulties of exact dosage* in the iontophoresis technique, it has not been possible to elucidate this problem in the present studies. MAGGIORA (1963) considered it possible to introduce 5 mg of serotonin into the skin of guinea pigs by iontophoresis at 2 mA for 10 minutes, but gave no biochemical determinations in controls. In the present work only about 40 / of the quantities obtained by calculation were in fact introduced. Presumably the fraction of the maximum quantity obtained by calculation which is actually introduced, is so variable under different experimental conditions that a biochemical or biological control will always be necessary The iontophoresis technique *is further limited to molecules with suitably low molecular* weight (ABRAMSON & GORIN 1940)

The acute unspecific inflammatory reaction in mice, which occurs after xylene, has previously been shown to develop in two phases, the initial plasma oedema being reabsorped after 24 hours, after which the water content of the tissue increases again, presumably as an expression of the regenerative processes of the tissue (LANGGÅRD HVIDBERG & SZPORNY 1964) The present work indicates that the effect of histamine as well as of serotonin occurs during the first of these phases. The oedema producing effect of serotonin is more intensive than that of histamine and occurs only with low concentrations.

Summary

Histamine and serotonin were introduced into the skin of mice by iontophoresis By biochemical and biological analyses it was shown that biogenic amines were introduced in this way not only into the loose interstitial connective tissue of the corium, but also into the subcutaneous tissue. The quantities introduced represented about 40 / of the maximum values obtained by calculation. By means of a special technique for marking and cutting out two symmetrical pieces of skin, one of which was treated, whilst the other one served as a control, quantitative data on the amount of amines introduced could be correlated to the size of the oedema produced at different times of the inflammatory response. The results were compared with those of a previous investigation, where the inflammatory reaction under identical conditions, was caused by xylene. It was seen that histamine as well as serotonin produce their effect in mice during the early phase of the inflammatory reaction, that the oedema producing effect of serotonin is more intense than that of histamine, though none of the amines examined play any quantitatively dominating role, and that serotonin may have an oedema forming effect in suitably low concentrations only

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On the Usefulness of the Term 'Per gram Fat free Solids"

By

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(Received October 3, 1966)

The quantity of a given substance in a given tissue can generally be stated only in relative values, i.e. in relation to the quantity of one or more of the other substances contained in the tissue. As the water and fat contents of most tissues can vary within wide limits, without otherwise influencing essentially their composition, a minimum spread of the biological material will be achieved in many cases when the quantities of the different substances are given in relation to the quantity of the dry defatted tissue. For this reason the term *per unit weight of fat free solids* has been increasingly used and is evidently regarded by many investigators as the ideal method for indicating the chemical composition of solid tissues and hence also of connective tissue. We are thus presenting some examples of comparative investigations showing that the term is often unsatisfactory and may even be the cause of serious misinterpretations. In order to demonstrate this, the contents of histamine in the skin under different experimental conditions has been determined but, as already emphasized, it is a question of considerations of general principle which could apply for any substance in any solid tissue.

Methods

The histamine content in the skin during the inflammatory reaction

Two symmetrical 5.5 cm^2 pieces of skin were marked on the depilated skin of the back of 4–6 week old mice under light halothane (fluothane ®) anaesthesia. In previous work it was shown that in untreated animals, these areas could be cut out – two and two with identical weight and composition (SZPORNY LANGGAARD & HVIDBERG 1964). An acute inflammatory reaction was induced within the limits of the right side area, by application of xylene for two minutes. Fifteen minutes, 2½ 24 or 48 hours later, the animals were killed

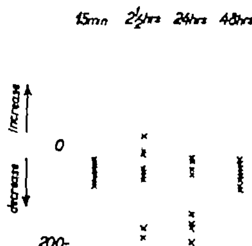


Fig. 1 Histamine content of inflamed skin in comparison with normal skin, expressed in µg per g of fat free solids, at different intervals after initiation of the inflammatory reaction

and exsanguinated. The two pieces of skin, including the subcutaneous tissue, were cut out, weighed, dried, defatted and the histamine content determined spectrofluorometrically in the fat-free solids, as condensation product of histamine and orthophthalaldehyde (cf. ZACHARIAE 1965). The values obtained for the contents of histamine, expressed in µg per g fat-free solids, were converted, by means of the total contents of fat-free solids on the two sides, to the total content of histamine on the two sides.

The histamine content in the corium and subcutaneous tissue

From the depilated back skin of 4–6 week old mice, which had previously been drained of blood, a well defined area measuring 5.5 cm² was cut out (corresponding to the left side of the above mentioned areas). As the subcutaneous tissue is easily scraped off the far firmer corium, the piece of cut skin was separated into corium and subcutaneous tissue each of which was weighed separately dried and defatted. After this, the histamine content was determined as described above for the corium as well as for the subcutaneous tissue. The analytical data of histamine content, expressed as µg per g fat free solids, were converted by means of the values for the total contents of fat-free solids in the excised corium and corresponding subcutis, to the total content of histamine in the excised parts of the corium and subcutaneous tissue

Results

Fig. 1 shows the difference in histamine content of inflamed skin and normal skin 15 minutes, 2½ 24 and 48 hours after the induction of the inflammatory reaction when the histamine content is given in relation to the quantity of fat free solids. The points indicate single observations. *The same observations* are given in fig. 2 but the data are here converted,

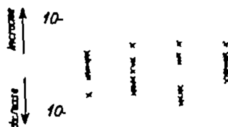


Fig. 2. The points indicate the same observations as in Fig. 1 but here each value has been converted to indicate the total histamine content in a piece of skin, 5.5 cm² large, in comparison with the corresponding non-inflamed skin.

Table 1

In four mice, defined piece of normal skin with surface area of 5.5 cm² was excised. The subcutaneous tissue was separated from the corium and the layers analyzed separately The table gives the weights of the two layers, their contents of fat-free solids and the histamine contents. The amounts of histamine are expressed as µg per g fat-free solids as well as the total amounts in the excised skin pieces.

Total weight (mg)		Fat-free solids (mg)		Histamine (µg/mg fat-free solids)		Histamine (total µg)	
Corium	Subcutis	Corium	Subcutis	Corium	Subcutis	Corium	Subcutis
150	116	44	14	0.40	0.52	20.0	6.4
133	117	42	17	0.52	0.63	21.8	10.7
112	92	35	12	0.43	0.52	15.1	6.2
125	113	43	15	0.48	0.51	22.2	6.6

as described above, to indicate the difference in the total histamine content between the two excised pieces of skin. Fig. 1 gives the impression of a distinct fall in histamine content of the skin during the development of the inflammatory reaction, whereas this is not evident from fig. 2.

Table 1 shows the weights in four mice, of the separated corium and subcutaneous tissue in defined pieces of skin with a surface area of 5.5 cm² as well as the corresponding values for the total weights of the fat-free solids and the total histamine content. In the same table the histamine contents of corium and subcutaneous tissue are given expressed in µg per g fat-free solids. The figures show firstly that whereas the corium weighs very little more than the corresponding subcutaneous tissue, the content

of fat-free solids are much higher in the corium. Secondly that although the histamine contents, indicated in µg per g fat free solids, are somewhat higher in the subcutaneous tissue than in the corium, the actual amount of histamine is 2–3 times higher in a piece of corium than in the corresponding piece of subcutaneous tissue.

Discussion

The usefulness of relative quantity indications in connection with comparative investigations depends on the constancy of the reference substance, also when this is made up of the fat-free solids. The term *per unit weight of fat free solids* is thus acceptable in principle, only when the quantity of fat free solids is unchanged during the experimental conditions in question or is the same in the two tissues that are being compared. The practical significance of the question is confirmed by the examples in this paper

It is generally assumed that histamine is liberated during the acute inflammatory reaction (surveys *Histamine Ciba Foundation Symposium.* 1956 NORN 1965 ZACHARIAE 1965) This assumption is particularly supported by the results shown in Fig. 1 However the histamine decrease which can be inferred from fig 1 is only fictitious. In reality the histamine content is not decreased, but owing to the solid content of the oedema fluid (SZPORNY LANGGÅRD & HVIDBERG 1964) the content of fat-free solids has risen. The observation naturally does not exclude the possibility that under other experimental conditions a disappearance of histamine may occur during the inflammatory reaction. Likewise it is possible that histamine exerts an effect on the development of the inflammatory process without being reflected in an alteration of the total histamine content. The example demonstrates, however that the fat-free solids should not be used as a reference substance in comparative investigations of normal and inflamed connective tissue.

The figures in table 1 which demonstrate the composition of a normal piece of skin show that the content of histamine in relation to the quantity of fat-free solids is higher in the subcutaneous tissue than in the corium, a finding that has sometimes been linked with the higher number of mast cells in the subcutaneous tissue. By conversion to total values it appears, however that the corium contains much more histamine than the subcutaneous tissue, in spite of the fact that the two layers are approximately of the same thickness. The most essential difference between the two layers of the skin is that the content of solids, expressed in percentage, is much higher in the corium than in the subcutaneous tissue chiefly because of the collagen content (LANGGÅRD 1965). For this reason substances which are

given in relation to the solids, have a lower level in the corium. The example shows that the fat-free solids should not be used as reference substance in cases of comparative investigations on tissues of different structures. The conclusion can also be extended to apply to comparative investigations on the same tissue from different species of animals or tissues from different sites in the same animals. In these cases the term *per unit weight of fat-free solids* should consequently not be to used either.

The term *per unit weight of tissue* (i.e. total tissue) which is also frequently used, has the same limitations as mentioned above. By way of example, it may be mentioned that the contrast between fig. 1 and fig. 2 would have been even more striking if the histamine content had been expressed in μg per g total tissue.

When tissue-analytical data are to be presented, a substance which is unaffected during the experimental conditions should be searched for. The other components of the tissue can then be indicated in relation to this substance. When dealing with the skin of small laboratory animals the different substances can with advantage be given, as in the present work, as the total amounts in a piece of skin defined by its surface area.

Summary

Examples are given in which the use of the term *per unit weight of fat free solids* may cause serious misinterpretations of analytical observations.

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The authors wish to thank Mrs. L. Jacobsen for her excellent technical assistance.

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In Vitro Studies of the Ciliotoxic Action of Ethanol Vapour in Relation to its Concentration in Tracheal Tissue

By

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(Received October 25 1966)

The ciliostatic action of respiratory irritant gases and vapours has frequently been investigated. In most cases the observations were confined to the relation between on the one hand, the gaseous concentration and the period of exposure to this, and on the other to the slowing or cessation of ciliary movement.

The available literature contains no reports on the tissue concentrations of ciliotoxic gases at the time of ciliostasis, although according to the first report on the effect of ethyl alcohol on ciliary motility published by PURKINJE & VALENTIN in 1835 it was evident that a certain concentration was required to produce a ciliostatic effect. In these experiments, which were made on oesophagus from *Rana Temporaria* and *Rana Esculenta*, it was found that when the specimens were immersed in 10% ethyl alcohol, ciliostasis occurred after 8 minutes, but this effect was not seen when a 1 % solution was used.

BREYER (1903) performed similar studies on frogs, using various alcohols in concentrations ranging from 5 N to 1/10 N and tissue sections measuring approximately 5 mm² on which the solutions were dropped. The interval required for ciliostasis varied with the concentration of the solutions. The alcohol concentrations in the tissues at the time of ciliostasis were, however not determined.

LOMMEL (1908) also studied the ciliostatic effect of alcohols. He reported that the ciliary activity in the respiratory mucosa of dogs ceased when certain quantities of alcohol were given orally The weight of the dogs was not stated.

The concentration of ethyl alcohol required to produce arrest of mucous flow was studied *in vitro* by HILL (1928) in various animal species. When fowl trachea was placed in a 10 % solution of alcohol in Ringer's solution the transportation of mucus ceased. A 5 % solution produced the same effect in rabbit trachea.

In contrast to Hill's results, PROETZ (1934) found that about 15 minutes immersion in 18 % ethyl alcohol in Locke's solution was required in order to produce ciliostasis in rabbit trachea. This effect was not obtained when a 15 % solution was used.

Postciliostatic resumption of ciliary motility was observed by BERNARD (1866) in oesophageal mucosa of frogs. The specimens were first exposed to ether vapour and then to fresh air. This was confirmed by ENGELMANN (1868), who demonstrated the same reversible course during exposure to ethanol vapour. He further noted a granular precipitation within the cells, which occurred at the same time as ciliostasis, after exposure to an unstated concentration of ethanol vapour. This precipitation disappeared when the tissue was exposed to fresh air. The ciliary movement then started again.

BREYER (1903) observed resumption of ciliary motility when exposure to alcohol was interrupted soon after ciliostasis occurred. He found that ciliostasis became irreversible if the exposure to alcohol was continued for some time after ciliostasis had started. From observations on ciliary motility for periods up to 48 hours the same investigator reported that the primary alcohols studied had an initial depressant action, which was followed by a phase of stimulation and finally by retardation of ciliary motility.

That ethanol vapour can accelerate ciliary movement was observed by ENGELMANN in 1868 in studies on vertebrates. The effect, however, was seen only when the tissue preparations were placed in hypertonic or hypotonic solutions of sodium chloride and not in isotonic solutions.

HILL (1928) found no accelerating action of ethanol on mucous flow. When absolute alcohol was applied to horse trachea, the transport of mucus ceased almost immediately, but when the alcohol had been washed off with Ringer's solution the cilia were still active. He believed this was probably due to a protective layer of coagulated mucin.

The effect of ethanol on pulmonary clearance was investigated by LAURENZI *et al.* (1963 & 1965 & 1966). Mice were exposed to an aerosol containing staphylococci. By killing the mice after fixed intervals and calculating the number of viable staphylococci in the respiratory mucosa, a measure of pulmonary clearance was obtained. Alcohol was injected intraperitoneally in doses related to the body weight, and in concentrations ranging from 5 to 21 %. The blood alcohol concentration was

determined as a four hour mean value. Coma inducing concentrations of alcohol in the blood (0 2–0.5 %) caused appreciable reduction of pulmonary clearance, and the higher the blood alcohol concentration, the lower was the pulmonary clearance.

The purpose of the present study was to observe the association between the concentration of ethanol vapour and the duration of exposure preceding cilliostasis in rabbit trachea *in vitro* and also to determine the concentration of ethanol in the tracheal tissue at the time of ciliostasis.

Methods

Ciliary motility was studied according to the method described by DALHAMN & LAGERSTEDT (1966) but specially modified for this investigation The principle of the method is that isolated, standardized preparations of trachea are exposed to various concentrations of alcohol vapour in special chamber During the exposure period, the ciliary motility is observed under light microscope and as soon as cilliostasis occurs the concentration of ethanol in the specimen is measured

Ethanol vapour in different concentrations was produced by conducting compressed air at constant flow rate through wash bottles containing alcohol and water in varying proportions 20 50 and 72 % v/v ethanol solutions were prepared by volumetric mixture of 95 % ethanol and distilled water Before the exposure was begun, the wash bottles, tubing and exposure chamber were warmed in water bath (fig. 1) to 36° The temperature of the compressed air varied with the room temperature and was about 21 Alcohol vapour was mixed with saturated water vapour at 36 in the proportion of 1 2. The flow of both vapours was measured with rotameters mounted on the wash bottles. The combined flow

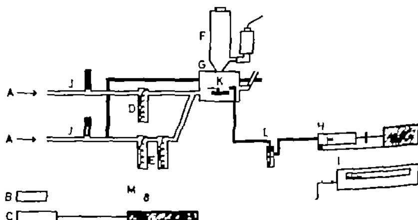


Fig. 1 Schematic drawing of the cilia motility studying unit. A. Compressed air B. Propeller for stirring water C Thermostatic controlled immersion heater D Ethanol in water solution. E. Distilled water F Microscope. G Exposure chamber H. Electrically driven syringe for gas-sampling. I Electrical thermometer J Rotameter K. Table for specimen. L. Impinger M. Water bath.

through the exposure chamber was approximately 12 litres/minute. Plastic tubing was used. The exposure chamber was made of glass and its volume was 31 ml.

The concentration of ethanol vapour in the exposure chamber was determined by gas chromatography on measured volumes of air which were collected from immediately above the specimen. The syringe used for this purpose had a constant rate of suction, which was maintained by an electric motor and was 10 % of the gas mixture flow in the chamber.

Tracheas were taken from rabbits which had been killed by injection of nembutal ® (mebumal NFN 100 mg). Circular sections of trachea 7, 9 or 13 mm in diameter and approximately 1 to 1.5 mm thick were punched out. They were stored in Deward dishes at saturated humidity and at 36°.

The specimens measuring 7 and 13 mm were used as two separate series. The specimens in the first series (7 mm) were exposed to ethanol according to a permutation programme in which the permutations included the individual rabbits as well as the time after which the rabbits were killed. In the other series the exposures were so made that 13 mm specimens from the same trachea were exposed to the same ethanol concentrations at different intervals after the rabbit was killed.

The ciliary activity in the centre of the specimen was observed by light microscopy and the time of ciliostasis was registered with a stopwatch. Immediately after the ciliary movement ceased, the specimen was lifted with forceps on to a glass for gravimetric determination of the weight. This averaged 91 mg in the 13 mm series, with a range of 128 to 67 mg. In the series of 7 mm specimens the weight ranged from 37 to 19 mg and the average was 27 mg.

After addition of 2 ml distilled water the specimens were exposed to ultrasound (90 kc.) in the same sealed glass for three minutes. The content of alcohol was thereafter determined by gas chromatography and the concentration of alcohol in the tissues was calculated.

For gas chromatography (Perkin-Elmer) a 3 mm × 1 m column was used with 20 % SE-30 Chromosorb V 60–80 mesh and a flame ionization detector. In order to estimate the evaporation of water from the specimens under the conditions of the experiments, a series of 7 mm sections was weighed both before and after the experimental procedure. The approximate weight loss was 11 % when the concentration of ethanol vapour at the time of ciliostasis was 9,000 ppm, 7 % at 22,500 ppm and 6 % at 26,900 ppm.

In control experiments we exposed 7 mm specimens to water vapour alone at the same humidities as were present according to theoretical calculations, in the preceding tests. This was accomplished by conducting compressed air at the same rate of flow through distilled water warmed so as to give saturated water vapour with the same partial pressures (theoretically calculated) as in the previous experiments. Five specimens were exposed to water vapour at each of the selected partial pressures.

The rate of ethanol absorption in the specimens was studied in a series of 7 mm specimens, which were exposed to various concentrations of ethanol. The ethanol concentration in the specimen was determined as already described, at various intervals from the start of exposure.

The significance of specimen size for the rate of ethanol absorption was studied in one series each of 7, 9 and 13 mm specimens. All specimens were exposed to 32,000 ppm ethanol vapour after which the ethanol concentration in the sepcimens was determined in the usual way.

Results

Figure 2 illustrates the results of the comparative experiments on 7 mm and 13 mm specimens. The relationship between the exposure time

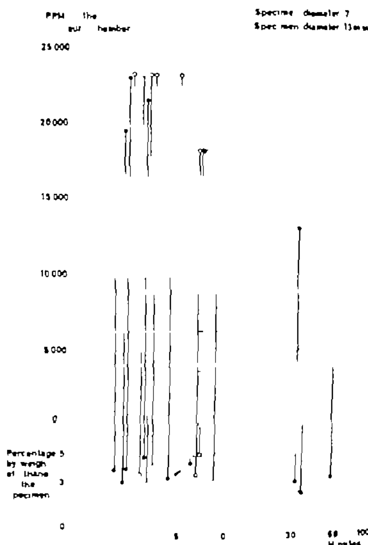


Fig. 2. Above relationship between atmospheric concentration of ethanol and exposure period before ciliostasis.
Below tissue concentration of ethanol at the time of ciliostasis.

preceding ciliostasis and the concentration of ethanol vapour in the chamber is shown in the upper part of the diagram, and the concentration of ethanol in the specimens at cilliostasis in the lower part. When ciliostasis had not occurred after 60 minutes the values were not included This was observed in four of the 13 mm specimens at 10 400 ppm ethanol vapour and in three of the 7 mm specimens at 9,500–11 000 ppm.

In the control specimens (7 mm) which were exposed to water vapour alone at the same theoretically calculated partial pressures as were used

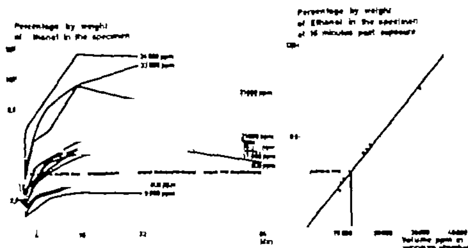
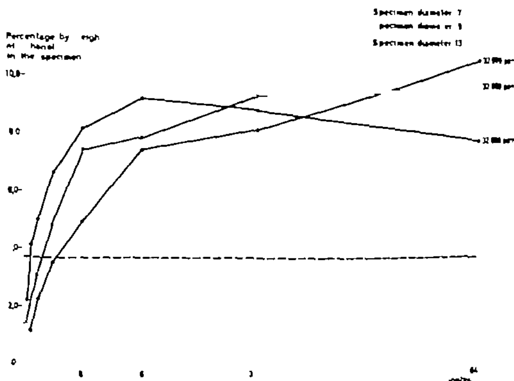


Fig. 3. Absorption rate of ethanol vapour in 7 mm specimens. Left ethanol absorption rate in the specimens ——— and tissue concentration of ethanol at ciliostasis – – – – – Right tissue concentrations of ethanol after 16 minutes of exposure to various concentrations of ethanol vapour

In the experiments with exposure to ethanol vapour ciliostasis occurred after a mean interval of 61 minutes at the lowest relative humidity (54 %), 100 minutes at intermediate humidity (73 %) and 117 minutes at the highest humidity (97 %).

The concentration of ethanol in the tissues at the time of ciliostasis averaged 3 8 / in the 7 mm specimens, with a range of 4 8 to 3.0 %. In the 13 mm specimens the mean was 3 6 / and the range 4.9 to 2.3 %.

Figure 3 presents the tissue concentrations of ethanol in 7 mm specimens after varying periods of exposure in the chamber and at different atmospheric concentrations of ethanol At the higher atmospheric readings, the rise in the tissue ethanol concentration was more rapid and the reading at equilibrium between chamber and tissue concentration was higher This equilibrium was reached after about 16 minutes at all chamber concentrations of ethanol. The tissue ethanol concentrations after exposure for 16 minutes to different chamber concentrations of the vapour are marked on the right in the diagram. It is seen that after 16 minutes the relationship between the tissue and chamber concentrations of ethanol could be expressed as a straight line. If the mean of the observed tissue concentrations of ethanol is interpolated in fig. 3 the lowest ethanol vapour concentration theoretically required to produce ciliostasis can be read as about 12,500 ppm. The periods of exposure preceeding ciliostasis at different chamber concentrations of ethanol can be read (left) from the points where the broken line of ciliostatic tissue concentration crosses the curve of absorption.



F g. 4 Specimen size and absorption rate. 7 9 and 13 mm specimens individually exposed to 32,000 ppm ethanol vapour

The variations in the pre-ciliostatic interval according to specimen size are shown in fig. 4 The 7 9 and 13 mm specimens were all individually exposed to 32,000 ppm ethanol vapour In the smaller specimens the concentration of ethanol rose more rapidly than in the larger ones Increase in specimen size from 7 to 13 mm entailed lengthening of the pre-ciliostatic interval from 1 to 4 minutes.

Discussion

In this investigation the tissue concentration of ethanol required to arrest tracheal ciliary activity *in vitro* was about 4 ‰ irrespective of the size and age of the specimen the atmospheric concentration of ethanol vapour and the duration of exposure preceding ciliostasis. Even when the atmospheric concentrations of ethanol produced ciliostasis after approximately the same time as occurred with water vapour alone in control specimens, the tissue concentration of ethanol was the same.

Ciliary motility in the ethanol-exposed specimens was observed for only one hour However since the tissue concentration of ethanol reached equilibrium with the gaseous phase after only about 16 minutes (fig. 4),

longer observation of the ciliostatic effect was not necessary in specimens of the sizes we used Since the rate of evaporation of water is dependent on the size of the specimen, it is essential to work with standardized specimen sizes in order to eliminate this source of error in the results.

The rate of absorption of ethanol vapour in the specimens was likewise dependent on their size (fig. 4) Absorption was more rapid in the smaller specimens. This further underlines the necessity of using standardized specimens in studies of this type on ciliary motility

The comparative brevity of the pre-ciliostatic intervals in this study was primarily attributable to the rate of water evaporation as a function of specimen size and the flow rate of the vapour in the exposure chamber The larger the specimens and the higher the humidity in the exposure chamber the longer the pre-ciliostatic interval. The relative humidities used in the present experiments ranged from about 54 / at the highest ethanol vapour concentrations, to 97 / at the lowest. The fluid loss from the specimens was about 11 / in the presence of ethanol vapour concentrations which produced ciliostasis after about one hour However the tissue concentration of ethanol at ciliostasis was independent of the humidity in the exposure chamber

These results cannot be directly compared with observations made by other investigators. Assuming that the specimens we used had a water content of about 50 /, the ratio of alcohol to water content was approximately 7–7 5 / at ciliostasis. The earlier observations which are most suitable for comparisons with our own are those published by BREYER (1903), HILL (1928) and PROETZ (1934) From BREYER s report it seems that the concentration of the solution which caused ciliostasis was of the order of 6 / and that the pre-ciliostatic interval was about 6 hours The solutions he used, however were not quite physiological. In HILL s experiments mucous flow ceased when rabbit tracheas were immersed in a 5 / ethanol solution. PROETZ reported ciliostasis after immersion of similar specimens for 15 minutes in an 18 / ethanol solution.

Reversible precipitation of proteins occurs in the presence of alcohol. The concentration of alcohol required to produce this effect is dependent on the nature of the protein. Engelmann (1868) observed precipitation in association with ciliostasis produced by alcohol but he did not report the strength of the alcohol solutions. HILL (1928) described a coagulating action of absolute alcohol on the mucin layer of horse trachea. PROETZ (1934) stated that when rabbit trachea was immersed in strong (20 /) alcohol solution fissures appeared due to coagulation and shrinkage Between the fissures ciliary motility persisted for several minutes.

The question arises whether the ciliostasis observed in our experiments involved true cessation of ciliary movement, or whether the ciliary

motility was masked by coagulated mucoprotein. The latter possibility was eliminated by the absence of discernible changes in the mucus. Thus after ciliostasis, for example the blood vessels were still clearly visible beneath the secretion

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Effect of Histamine Liberators on Rat Peritoneal Mast Cells and Influence of Antirheumatic Agents

By

Svend Norn

(Received October 14, 1966)

When peritoneal mast cells or mesenteric mast cells from sensitized rats are incubated *in vitro* with the specific antigen, histamine is liberated (UVNÄS & THON 1959 MOTA & ISHII 1960 PERERA & MONGAR 1963 NORN 1965). If, however towards the end of the sensitization period, the rats are treated with various antirheumatic agents, this liberation of histamine may be inhibited (NORN 1965) The mechanism of this action by antirheumatic agents is unknown. The inhibition may be due to an inhibited antibody production, an inhibited antigen-antibody reaction, or an inhibition of the enzymatic processes which are assumed to be elicited in the mast cell during antigen-antibody reactions (MOTA & ISHII 1960 UVNAS 1962)

The liberation of histamine caused by compound 48/80, lecithinase A (phosphatidase A or phospholipase A) and α-chymotrypsin is influenced by certain factors in a manner similar to the histamine liberation in antigen-antibody reactions (UVNÄS 1962 UVNÄS 1963 UVNÄS & ANTONSSON 1963). It is likely therefore, that the histamine liberators and the antigen-antibody reaction liberate histamine by eliciting the same enzymatic mechanism in the mast cell (UVNÄS 1962 UVNAS & ANTONSSON 1963). The object of the present study was to throw further light on this theory and to investigate the possibility of determining – on the basis of the results obtained by experiments on histamine liberators – the mode of action of antirheumatic agents on the histamine liberation elicited in an antigen-antibody reaction.

Experiments and Method

The experimental animals were adult female albino rats weighing between 150 and 180 g. The rats were injected subcutaneously for 3 days with 100 mg/kg hydrocortisone, 200 mg/kg phenylbutazone or 25 mg/kg sodium aurothiosulphate (test animals). The dose was administered in a volume of 10 ml/kg body weight, the last-mentioned substance being administered in aqueous solution and the other substances in the form of suspension. The suspension medium consisted of 1 part of Acacia Mucilage (U.S.P. 47) and 6 parts of water.

18 ml of a 37° modified Tyrode solution (1.44×10^{-1} M NaCl, 3×10^{-3} M KCl, 8×10^{-4} M $CaCl_2$, 8×10^{-3} M Na_2HPO_4, and 3×10^{-3} M KH_2PO_4) was injected intraperitoneally into these rats as well as into the untreated rats (controls), the test rats being injected 1½ hours after the last administration of the antirheumatic agent. The rats were killed by bleeding from the carotid arteries, 30 sec. after the injection of the modified Tyrode solution. The peritoneal cavity was opened and emptied of fluid. As a rule this yielded about 12 ml (peritoneal mast-cell suspension, *PMS*).

Dose-response curve of the histamine liberators

PMS from several controls was pooled. From this pool samples of 3.50 ml were removed, and compound 48/80, polymyxin B, lecithinase A, or α-chymotrypsin in various concentrations were added (fig. 1). As blanks, we used the corresponding samples without the addition of histamine liberator. The samples were incubated for 10 min. at 37°. The quantity of histamine liberated from the mast cells was then determined. The specimens were centrifuged at 1500 x g for 45 min., and 2.50 ml of the mast-cell-free supernatant was removed. The protein content of the supernatant was precipitated by adding 200 µl 3.9 N-$HClO_4$, and centrifugation was repeated (1500 × g for 10 min.). In 2.00 ml of the protein-free supernatant the content of histamine was determined by the method of SHORE *et al.* (1959).

This method was used with negligible modifications. The fluorescence at 470 mµ resulting from activation at 365 mµ (B Hg I filter) was measured in a spectrofluorometer (Photovolt Model 540).

To calculate the percental histamine release, 2 samples of 3.50 ml were removed from the pooled PMS for determination of total histamine content. In order to liberate the intracellular histamine 550 µl of 3.0 N $HClO_4$ was added. The histamine content was determined as previously in 2.00 ml of the protein-free supernatant. The quantity of released histamine was given in per cent of the total content, the liberated as well as total content being corrected for the amount of histamine released by the mechanical manipulation (blank sample).

Significance of mast cell count in PMS

From the dose-response curves (fig. 1) we can obtain, for each histamine liberator, the concentration which gives a histamine liberation of about 50–80% of the total content in the sample (EC50–80). The histamine liberators were used in these concentrations partly in PMS from controls and partly in dilutions of this PMS with modified Tyrode solution in the ratio 1 + 1 and 1 + 2 (table 1). After incubation for 10 min. at 37° the liberated quantity of histamine was determined in per cent of the total content of the sample.

Duration of histamine liberation

Samples of PMS from control rats were incubated at 37° for 2, 10, and 30 min. respectively with compound 48/80, polymyxin B, lecithinase A, or α-chymotrypsin. These substances were used in the concentration EC50–80. Thereafter the specimens were centrifuged by 6. The histamine release was given in per cent of the total content of the sample (table 2).

Influence of antirheumatic agents on histamine liberation

From the PMS of each rat (test as well as control rats) two samples f 3.50 ml were removed. One sample was incubated for 10 min. at 37° with compound 48/80, polymyxin B, lecithinase A, or α-chymotrypsin, in the concentration EC50–80 (table 3). The amount of histamine liberated in this sample was determined. I the ther sample the total histamine content was determined. Table 3 shows the histamine release in samples from the test rats and in the controls. The inhibition was calculated according to the formula $Pc-Pt/P \times 100\%$, where Pc and Pt are the average proportion of liberated histamine in samples from control and test rats. The values given for the inhibition are based on mean values f determinations in 6 test and 6 control rats. Because of day-to-day variations in the histamine hberation, *samples from the test rats were always compared with samples from control rats from the same day*

Results

Dose-response curves of the histamine liberators

As is apparent from fig. 1 all the substances studied showed liberation of histamine *in vitro* increasing with an increasing concentration of the histamine-liberating substance. It would seem reasonable to consider the middle part of the curves as rectilinear Compound 48/80 showed the greatest histamine-liberating effect, since in concentrations of about 0 1 µg/ml of peritoneal mast-cell suspension, it released about 50 / of the

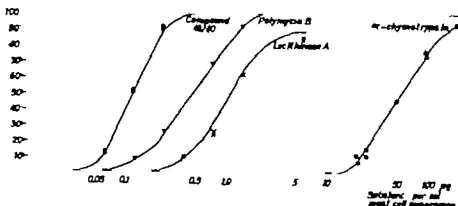


Fig. 1 Release of histamine from suspensions of rat peritoneal mast cells caused by arious concentrations of compound 48/80, polymyxin B, lecithinase A, or α-chymotrypsin.

Ordinate Release f histamine in per cent of the total contents of the samples taken from mixture of peritoneal mast cell suspensions.

Abscissa Concentration of compound 48/80, polymyxin B, lecithinase A or α-chymotrypsin in the sample (µg per ml sample).

Each point represents single determination.

total histamine content of the sample. To obtain the same effect with polymyxin B, lecithinase A, and α-chymotrypsin, the concentrations have to be 4, 10, and 500 times greater respectively. In sufficiently high concentrations compound 48/80 and polymyxin B will release all the histamine in the mast-cell suspension.

Significance of mast-cell count in PMS

It is evident from table 1 that in the three samples with different mast-cell counts per ml of sample, a constant percentage of the total histamine content of the sample was liberated on incubation with each of the histamine liberators studied.

Duration of histamine liberation

Table 2 shows that in the course of 2 min. all the histamine liberators released practically the entire amount of histamine which is liberated in 10 min. and that no further quantity could be released by incubation for 30 min. than for 10 min.

Influence of antirheumatic agents on the histamine liberation

Pre-treatment of the rats with hydrocortisone did not reduce the *in vitro* histamine release elicited by compound 48/80, polymyxin B, lecithinase A, or α-chymotrypsin (table 3).

Table 1

Release of histamine by compound 48/80, polymyxin B, lecithinase A, or α-chymotrypsin in samples taken from the same mixture of rat peritoneal mast cell suspensions. The release of histamine is expressed in per cent of the total content of the sample. The concentrations of the substances are given as µg per ml sample. The samples were used either undiluted or diluted (1 + 1 or 1 + 2) with a modified Tyrode solution. Duplicate determinations per sample are given.

Dilution of a suspension of peritoneal mast cells	Histamine release in per cent			
	Compound 48/80 0.1[illegible] µg/ml	Polymyxin B 0.50 µg/ml	Lecithinase A 1.50 µg/ml	α-Chymotrypsin 100 µg/ml
Undiluted	46 59	62 57	79 79	60 76
1 + 1	58 58	55 54	77 83	73 70
1 + 2	57 59	53 63	78 78	71 71

Table 2

Duration of histamine release from samples of suspensions of rat peritoneal mast cells by compound 48/80, polymyxin B, lecithinase A, or α-chymotrypsin. The release of histamine is expressed in per cent of the proportion liberated by incubation for 30 minutes.

Duration of incubation in minutes	Histamine release in per cent of the histamine released in 30 minutes			
	Compound 48/80 0.125 μg/ml	Polymyxin B 0.50 μg/ml	Lecithinase A 1.50 μg/ml	α-Chymotrypsin 100 μg/ml
2	98	86	99	86
10	98	100	100	102

Table 3

Inhibit ry effect of antirheumatic agents on the release of histamine caused by compound 48/80, polymyxin B, lecithinase A or -chymotrypsin. Rats (test group) were pre-treated for three days with a daily subcutaneous injection of the antirheumatic agent and killed 90 minutes after the last dose. Suspensions of mast cells derived from the peritoneal cavity of pre-treated as well as of untreated rats (control group) were incubated with ne of the histamine-liberating substances. The substances are given in the table as μg per ml of suspension. The inhibitory effect is expressed in per cent of the histamine release in the control group.

Pre-treatment for 3 days with antirheumatic agents	I hibitory effect f antirheumatic gents in % on histamine release in control group after:			
	Compound 48/80 0.125 μg/ml	Polymyxin B 0.50 μg/ml	Lecithinase A 1.50 μg/ml	α-Chymotrypsin 100 μg/ml
Hydrocortisone 100 mg/kg	6	0	0	0
Phenylbutazone 200 mg/kg	0	0	15*) 26)	0
Sodium aurothio-sulphate 25 mg/kg	8 19	18	22) 28*)	70)

) $p < 0.05$
) $p < 0.01$ by t-test

Sodium aurothiosulphate significantly inhibited the histamine release caused by α-chymotrypsin and lecithinase A. This inhibition amounted to 70 / ($P < 0.01$ in the t-test) and 25% ($0.01 < P < 0.05$) respectively The histamine liberation caused by compound 48/80 and polymyxin B

was not significantly influenced by pre-treatment with sodium aurothiosulphate.

Pre-treatment with phenylbutazone showed an inhibition of 20% ($0.01 < P < 0.05$) of the histamine release elicited by lecithinase A, while the release caused by the other histamine liberators remained unaffected.

None of the antirheumatic agents used altered the total content of histamine in the PMS, samples from test as well as from control rats showing a content of about 1 µg histamine base per ml of sample (i.e. no significant difference in the content $P > 0.05$).

Discussion

It is well-known that the sensitivity of mast cells to a given histamine liberator varies within wide limits from species to species, even when mast cells from the same site are studied. Even within the same species large variations in histamine liberation from the peritoneal mast cells have been reported by various investigators using different techniques.

By using a suspension of cells from the peritoneal cavity of the rat instead of isolated mast cells, the present author in this study obtained a release of histamine by compound 48/80 which corresponds to the findings of BRAY & ARSDEL (1961). Thus, compound 48/80 in a concentration of 0.1 µg/ml cell suspension, induced liberation of about half the total histamine content of the sample and in higher concentrations the entire content was released.

UVNÄS & THON (1959) who did not use a peritoneal cell suspension, but peritoneal mast cells isolated by differential centrifugation found that in some cases the histamine liberation by compound 48/80 was far less than that found in the present study. Presumably this is a question of an altered sensitivity of the mast cells to this histamine liberator. However the altered sensitivity cannot be caused by differences in the ionic composition, temperature, or pH, as these factors were identical in UVNÄS & THON's study and in the present one. It is possible that only the more resistant mast cells force their way down into the lower phase, but it is more likely that the sensitivity of the mast cells alters whilst in the lower phase. This is indicated by the fact that UVNÄS & THON was able to normalize the sensitivity of the isolated mast cells by adding a small quantity of serum or albumin to the lower phase (UVNÄS & THON 1959 & 1961). They could not, however, obtain a 100% release of histamine by compound 48/80 as was found in the present study. This may mean that the cells did not after all attain their complete biological activity. Therefore, in experiments of this nature, it seems better to use *non-isolated* mast cells.

In the present study the histamine release was investigated not only for compound 48/80 but also for polymyxin B lecithinase A, and α-chymotrypsin. Compound 48/80 was found to exert the greatest histamine-liberating activity the activity of the other substances decreasing in the order given. This is in keeping with the findings of UVNÄS & ANTONSSON (1963)

With all the substances studied the histamine release proved to be a rapid process, since almost the entire liberated histamine content was released within 2 minutes. This is in accordance with studies on compound 48/80 (UVNÄS & THON 1961).

As reported by BRAY & ARSDEL (1961) the proportion of liberated histamine was found to be independent of the number of mast cells in the cell suspension (table 1) The variations in the mast cell count from cell suspension to cell suspension, is thus, of no significance for the histamine release.

UVNÄS and his associates assume that the same enzyme processes are responsible for the histamine release elicited by different histamine liberators, and that these enzyme processes are also elicited in an antigen antibody reaction. These views are based indirectly on the fact that the histamine release caused by the various histamine liberators and by the antigen-antibody reaction are influenced in the same way by factors such as temperature, pH, ionic content, and enzyme inhibitors (UVNÄS 1962 UVNÄS 1963 UVNÄS & ANTONSSON 1963)

It is remarkable that the histamine release by α-chymotrypsin is inhibited if the rats are pre treated with sodium aurothiosulphate, and that the histamine release caused by lecithinase A is inhibited by pre-treatment with sodium aurothiosulphate or with phenyl butazone (table 3). The only reasonable explanation is that sodium aurothiosulphate is able to block the enzymatic processes involved in the histamine release caused by lecithinase A or α-chymotrypsin, and that phenylbutazone is able to inhibit those enzymatic processes which are involved in histamine release caused by lecithinase A.

Since the histamine release caused by the 4 histamine liberators studied in equipotent concentrations is influenced in different ways by pre-treatment with the same antirheumatic agents, viz. sodium aurothiosulphate or phenylbutazone, it may be concluded, that the various histamine liberators cannot have elicited the same enzymatic processes.

The finding that hydrocortisone, for example does not influence the histamine liberation caused by the histamine liberators investigated (table 3) while hydrocortisone in the same dose gives a significant inhibition of the histamine release in an antigen-antibody reaction (NORN 1965), can be explained as follows Either the enzyme processes elicited

in an antigen-antibody reaction are different from those elicited by the histamine liberators, or hydrocortisone does not influence the enzymatic processes during the antigen-antibody reaction, but at an earlier stage, e.g. during the antibody production or during the antigen-antibody binding. Similar conclusions may be drawn for phenylbutazone and sodium aurothiosulphate with regard to some of the histamine liberators investigated (cf table 3) On the other hand, no positive conclusions can be drawn as to whether the histamine release caused by lecithinase A has one or more enzymatic links in common with the antigen-antibody reaction, on which phenylbutazone and sodium aurothiosulphate may act, and whether the histamine release caused by α-chymotrypsin has an enzymatic link in common with the antigen antibody reaction on which only sodium aurothiosulphate has an inhibitory effect. The fact is that the above named antirheumatic agents may easily be assumed, to inhibit different enzyme processes in the different forms of histamine release or to inhibit the antigen-antibody reaction itself

On the basis of the available data, we can only conclude that if hydrocortisone inhibits the histamine release in the antigen-antibody reaction by an inhibitory effect on the enzyme processes elicited in the mast cell in this reaction, these enzyme processes must differ from those elicited by the histamine liberators Similar views may be advanced with regard to the effect of the other antirheumatic agents on the action of some of the histamine liberators investigated.

Summary

The release of histamine in a suspension of non-isolated peritoneal mast cells from the rat was studied, following incubation *in vitro* with compound 48/80, polymyxin B lecithinase A or α-chymotrypsin. The dose-response curves were approximately rectilinear Compound 48/80 was found to exert the most potent histamine-liberating effect and α-chymotrypsin the weakest effect. With compound 48/80 the entire content of histamine could be liberated. With all histamine liberators the release of histamine occurred within 2 minutes, and was independent of the number of mast cells in the cell suspension. The investigations on compound 48/80 indicate that the biological activity of the mast cells is apparently better preserved in a suspension of non-isolated peritoneal mast cells than in isolated mast cells.

Studies on the inhibition of the histamine liberation after pre-treatment of the rats with various antirheumatic agents indicate that the various histamine liberators release histamine by eliciting various enzyme pro-

cesses in the mast cell. It is still not known whether any of the histamine liberators do in fact release histamine by eliciting the same enzymatic mechanism as in the antigen-antibody reaction.

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Studies on the Subcutaneous Absorption in Mice
II Influence of Toxicity on the Dynamics of Subcutaneous Absorption

By

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(Received October 4 1966)

Recently SECHER HANSEN LANGGÅRD & SCHOU (1967) described a method by which it was possible to study the disappearance rate of water and solutes from a subcutaneous "depot" in mice and at the same time to measure the decrease of the volume injected.

The influence of different osmotic concentrations on the absorption rate of water and solutes injected subcutaneously was studied by the use of this method.

Method

Albino, male mice of the Leo Strain strain, 4–6 weeks old were used and were maintained on standard laboratory diet and with free access to water

Some of the experimental procedures have previously been described in detail (SECHER-HANSEN, LANGGÅRD & SCHOU 1967a). While the animals were under light halothane (fluothane ®) anaesthesia, two symmetrical areas were marked on the depilated skin of the back. A standard volume of 80 µl of one of the following aqueous solutions was injected subcutaneously within the right-side area Sucrose, 6, 10 or 14 %, NaCl, 0.9 or 1.2 % or distilled water all containing approximately 3 µc $^{3}H_2O$ (New England Nuclear Corp., Boston) or ^{14}C-sucrose (The Radio Chemical Centre Amersham) per dose. In parallel series of experiments the above mentioned solutions contained 40 I.u. of hyaluronidase (penetrase Leo ®) per dose.

In each animal the remaining part of the volume injected (measured as the difference in weight between the injected and the un-injected piece of skin) and the remaining radioactivity were determined as described by SECHER-HANSEN, LANGGÅRD & SCHOU (1967a) 5 and 15 minutes after the injections.

Results

Figure 1 shows the percentual reduction of a subcutaneously injected volume of 80 µl of 6, 10 and 14 % solutions of sucrose either without or with 40 units of hyaluronidase per dose. In the course of the first 15 minutes after the injections, the depot of a 10 % sucrose solution decreased slightly whereas the depot of a 14 % sucrose increased significantly. Experiments with 6 and 10 % sucrose solutions showed identical results. Initially a more rapid decrease of the volume of 6 % sucrose than of 10 % sucrose ($p < 0.01$) was seen when hyaluronidase was added to the solutions injected. An increase in weight of the injected pieces of skin was

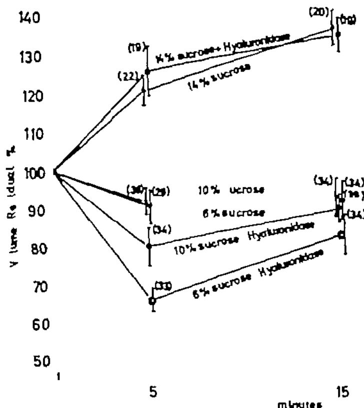


Fig. 1 Ordinate Residual volume of 80 µl of 6, 10 or 14 % sucrose in water with or without 40 i.u. of hyaluronidase per dose, 5 and 15 minutes (bscissa) after subcutaneous injections into the right of two symmetrical areas (3.5 cm²) of depilated skin of the back of mice. The residual volumes are determined as the difference in weight between the injected and the uninjected sides, and are expressed in per cent of the 80 µl injected. The standard errors of the means are indicated by vertical lines. Number of experiments are given in brackets.

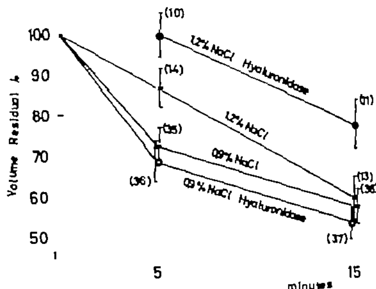


Fig. 2. Ordinate Residual volume of 80 μl of 0.9 or 1.2 % NaCl in water with or without 40 units of hyaluronidase per dose, 5 and 15 minutes (abscissa) after subcutaneous injections into the right of two symmetrical areas (3.5 cm²) of depilated skin of the back of mice. The residual volumes are determined as the difference in weight between the injected and the uninjected sides, and are expressed in per cent of the 80 μl injected. The standard errors of the means are indicated by vertical lines. Number of experiments are given in brackets.

observed for all the solutions from 5 to 15 minutes after the injections. For the solution containing 6 % sucrose with hyaluronidase, this rise was statistically significant ($p < 0.01$)

Figure 2 shows the percentual reduction of standard volumes of 1.2 and 0.9 % NaCl solutions. Comparing fig. 1 and fig. 2 it is seen that the volume of physiological saline decreases at a greater rate than a volume of "isoosmotic" sucrose solution (i.e. 10 %). Further fig. 2 shows that there is no significant difference between the decrease in volume of a 1.2 % NaCl and a 0.9 % NaCl solution. When hyaluronidase is added, a significantly lower rate of decrease appears for the former solution ($p < 0.01$). It is finally seen (fig. 2) that a volume of 1.2 % NaCl containing hyaluronidase, decreased at a lower rate than a corresponding volume of 1.2 % NaCl but without hyaluronidase. These volumes decreased during the observation period from 5 to 15 minutes after subcutaneous injections.

Figure 3 shows the residual volumes 5 and 15 minutes after subcutaneous injections of 80 μl of distilled water with or without hyaluronidase. Comparing fig. 1 and 3 it is seen that a volume of water decreases at approximately the same rate as volumes of 6 % sucrose and 10 % sucrose. Addition of hyaluronidase increases the disappearance rate significantly

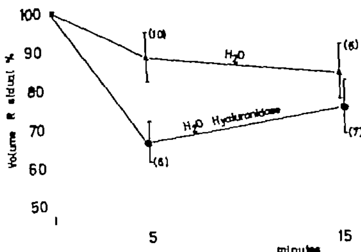


Fig. 1. Ordinate Residual volume f 80 µl of distilled water with or without 40 units of hyaluronidase per dose, 5 and 15 minutes (abscissa) after subcutaneous injections I to the right of two symmetrical areas (5.5 cm²) of depilated skin of the back of mice. The residual volumes are determined as the difference i weight between the injected and the uninjected sides and are expressed in per cent of the 80 µl injected The standard errors of the means re indicated by vertical lines. Number of experiments are given in brackets.

and the residual volume corresponds to the residual volume of a 6 % sucrose solution containing hyaluronidase.

In table 1 the residual ^3H_2O- and ^{14}C sucrose radioactivity is shown (in per cent of the total injected dose) 5 and 15 minutes after injections of standard volumes of distilled water 0.9 % NaCl, 6, 10 or 14 % sucrose solutions. In table 2 corresponding data are presented for the same solutions with the addition of hyaluronidase. In all cases the disappear ance rate for the water molecules was significantly faster than for the sucrose molecules. If the solutions did not contain hyaluronidase, the disappearance rate of labelled water and sucrose molecules was faster in the isoosmotic sodium chloride solution than in the other solutions. After addition of hyaluronidase, the disappearance rates of ^3H_2O and of ^{14}C sucrose increased in all the solutions, (except in the physiological sodium chloride) so that the disappearance rates were equal to the rate of disappearance in physiological sodium chloride solution

Discussion

There are few quantitative determinations on the absorption of hyper and hypo-osmotic solutions from the subcutaneous tissue. Based on

Table 1

Residual radioactivity due to $^{3}H_2O$ or ^{14}C-sucrose, 5 and 15 minutes after subcutaneous injections of 80 µl of 0 9 % NaCl, 6, 10 14 % sucrose or distilled water into the right of 2 symmetrical areas (5.5 cm²) of depilated skin on the back of mice. The residual radioactivity was determined as the difference in the total radioactivities between the injected and the uninjected sides, and expressed in percent of the total injected dose, measured as the radioactivity of an excised skin sample, injected with the standard dose and corrected for background counting. Figures in brackets indicate number of experiments.

	$^{3}H_2O$		^{14}C-sucrose	
	5 min. mean ± s.e.m.	15 min. mean ± s.e.m.	5 min. mean ± s.e.m.	15 min. mean ± s.e.m.
0.9 % NaCl	22 ± 2.4 (16)	3.3 ± 0.60 (17)	47 ± 3 1 (9)	16 ± 1.2 (9)
6 % sucrose	37 ± 2.3 (11)	4.5 ± 0.33 (15)	75 ± 2.3 (9)	39 ± 2 0 (9)
10 % sucrose	39 ± 2.2 (15)	4.1 ± 0.43 (11)	69 ± 2.4 (13)	38 ± 2.3 (13)
14 % sucrose	31 ± 3.2 (8)	5.6 ± 1.21 (9)	69 ± 1 7 (11)	32 ± 1 6 (9)
Distilled water	41 ± 4 1 (6)	5.1 ± 1.32 (6)		

studies on the absorption from the pleura and the peritoneum it is generally believed that non isoosmotic solutions injected subcutaneously are primarily made blood isotonic by water movements between the depot and the surrounding tissue. The depot should then be absorbed in the same manner as a primarily isotonic solution (PUTNAM 1923 DANOWSKI, WINKLER & ELKINTON 1947 WEBB *et al* 1950 ABBOT *et al* 1952). It is, however questionable whether comparisons can reasonably be made between absorption from cavities, and from the subcutaneous tissue which has specific physico-chemical properties. The results of the present work indicate that absorption from subcutaneous tissue follows quite special principles which seem to depend on properties of the loose connective tissue ground substance

Only for the hypertonic sucrose solution will the conditions correspond to the pleural absorption The sucrose solution injected subcutaneously will draw water from the surroundings (fig. 1) as would be expected on the basis that osmotic difference in pressure has to be eliminated. On the other hand, a volume of hypotonic sucrose solution will disappear at exactly the same rate as an isotonic solution (fig. 1) although an initial

Table 2

Residual radioactivity due to ^3H_2O or ^{14}C-sucrose, 5 and 15 minutes after subcutaneous injections of 80 µl of 0.9 % NaCl, 6, 10, 14 % sucrose or distilled water *containing 40 I.U. of hyaluronidase per dose* into the right of 2 symmetrical areas (5 5 cm²) of depilated skin on the back of mice. The residual radioactivity was determined as the difference in the total radioactivities between the injected and the uninjected sides, and expressed in per cent of the total injected dose, measured as the radioactivity of an excised skin sample, injected with the standard dose and corrected for background counting. Figures in brackets indicate number of experiments.

	^3H_2O		^{14}C-sucrose	
	5 min. mean ± s.e.m.	15 min. mean ± s.e.m.	5 min. mean ± s.e.m.	15 min. mean ± s.e.m.
0.9 % NaCl	18 ± 1.2 (16)	1.9 ± 0.36 (19)	40 ± 1.6 (10)	14 ± 1.6 (8)
6 /ₒ sucrose	23 ± 2.6 (14)	2.4 ± 0.36 (18)	43 ± 2.7 (10)	15 ± 1.2 (8)
10 % sucrose	26 ± 1.7 (11)	3.2 ± 0.37 (14)	50 ± 2.4 (14)	16 ± 1.3 (14)
14 sucrose	1 ± 1.4 (7)	2.6 ± 0.40 (7)	45 ± 2.2 (9)	14 ± 1.6 (8)
Distilled water	24 ± 2.4 (6)	3.2 ± 0.74 (7)		

marked absorption of the surplus water would have been expected Even a volume of pure water will disappear at the same rate as a corresponding volume of a 10 / sucrose solution (fig. 3). Considering the waterbinding properties of the connective tissue ground substance (HVIDBERG 1962), it seems likely that the surplus water molecules in the hypotonic solution can temporarily be *osmoticall) inactivated.* This explanation is substantiated by the fact that the difference in absorption rate expected, but not actually observed, between the hypotonic and the isotonic sucrose solution appears after the addition of hyaluronidase to the solutions (fig. 1). In this way the hyaluronic acid is depolymerized, and the water binding capacity destroyed locally

With regard to the hypertonic sucrose solution, the osmotic surplus is due to sucrose molecules, for which the ground substance has no binding capacity Hence water is drawn in from the surroundings. This state of affairs is not affected by addition of hyaluronidase. The ground substance may however also exercise an "osmostatic" action towards hypertonic solutions, as is apparent when a subcutaneously injected depot

of 1.2 / NaCl in water does not disappear at a significantly lower rate than 0.9 / NaCl in water Here it must be assumed that part of the surplus sodium ions will be "bound" to the anionic polyelectrolytes of the tissue (LANGGÅRD 1965) causing a reduction of the osmotic effect. This explanation is supported by the fact that after addition of hyaluronidase to the two solutions mentioned a significant difference is found between their rates of disappearance.

It was shown in a previous publication (SECHER HANSEN, LANGGÅRD & SCHOU 1967a) (and confirmed in this paper fig. 1 tables 1 and 2) that water molecules injected subcutaneously are exchanged by water molecules from the bloodplasma at a considerably higher rate than that corresponding to the disappearance of the volume injected. Practically all the water molecules originally injected have been exchanged 15 minutes after the subcutaneous injection, even in the solutions where the total volume surplus still remains at the injection site. In the paper mentioned it was further shown that water molecules are exchanged at a higher rate from 0.9 / NaCl than from 10 / sucrose, and it was suggested that sucrose molecules move more slowly than water molecules in the ground substance of the connective tissue. These results are confirmed in this work (tables 1 and 2) Moreover it has now been shown within the limits examined, that the rates of disappearance of water and sucrose molecules from an injected sucrose solution are independent of the osmolarity of the solutions (table 1)

When osmotic differences cause water movements, the equilibration is obtained mainly within the first 5 minutes. This is in accordance with the rapid exchange of water molecules. This exchange, as far as the sucrose-containing solutions are concerned, takes place at a higher rate after addition of hyaluronidase (table 2) This can be explained by the spreading effect of the hyaluronidase (cf SCHOU 1961) In view of this it is surprising that the addition of hyaluronidase does not affect the disappearance rate of water molecules from the sodium chloride solutions (table 2).

In this connection it is also remarkable that the increased disappearance rate of water and sucrose molecules obtained by addition of hyaluronidase to the sucrose solutions never exceeds the disappearance rates from a sodium chloride solution (table 1 and 2) The sodium chloride solutions in themselves apparently exert an effect similar to that of hyaluronidase. It is likely that a spreading factor in the tissue is activated by the NaCl injected (MEYER 1947 MEYER & RAPPORT 1952) The optimal concentration for the activation of the enzyme hyaluronidase by sodium chloride is just about that used here (MADINAVEITIA & QUIBELL 1941).

Water movements caused by osmotic differences seem, as mentioned above, to occur within the initial 5 minutes. The explanation of the latter

increase of the residual volume observed for several of the solutions from 5 to 15 minutes after the subcutaneous injection should be sought elsewhere (fig. 1) Experiments with intravenously injected ^{131}I human serum albumin have demonstrated the development of an inflammatory oedema during this period. Quantitative determinations of this aseptic inflammation caused by the trauma of the injections are being investigated (cf SECHER HANSEN LANGGÅRD & SCHOU 1967b).

Summary

Using a method previously described, the subcutaneous absorption of 80 µl of aqueous solutions of 0 9 / or 1.2 / NaCl, 6, 10 or 14 / sucrose or distilled water containing ^3H_2O or ^{14}C-sucrose molecules, without or with 40 I.u. of hyaluronidase per dose, was investigated.

It is shown that a surplus of water in hypotonic solutions can be osmotically inactivated by the ground substance of the connective tissue, which also exerts an "osmostatic" effect on electrolytes in a hypertonic solution, but there is no similar effect on non-electrolytes. It is further demonstrated that the rate of disappearance of water and sucrose molecules from an injected sucrose solution are independent of the tonicity of the solution Finally it seems probable that sodium chloride solutions in themselves have an effect similar to hyaluronidase on the disappearance rate of water and sucrose molecules.

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Studies on the Subcutaneous Absorption in Mice
III Aseptic Inflammation following Subcutaneous Injections*

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(Received October 14, 1966)

Investigations on the significance of the connective tissue ground substance in subcutaneous absorption (SECHER HANSEN LANGGÅRD & SCHOU 1967a & b) have made it desirable to determine quantitatively the inflammatory reaction caused by the injection trauma. The significance of the injected volume and the tonicity of the injected solutions have thus been determined. Furthermore the effect on the oedema formation of addition of hyaluronidase to the solutions has been investigated. The extent of the inflammatory reaction has been measured by the local uptake of circulating ^{131}I-human serum albumin

Methods

White male mice (Leo, Strain), 4–6 weeks old were used. Half the number of animals were injected subcutaneously with oestradiol monobenzoate 1 µg in 0.1 ml arachis oil, 6 and 4 days before the experiments. In this strain of mice oestradiol increases the amount of ground substance and the degree of polymerization of the hyaluronic acid.

Fifty µl of 5 % ^{131}I human serum albumin containing about 1.5 µc was injected into tail vein. After distribution period of 5 minutes, 80 µl of one of the following aqueous solutions was injected subcutaneously on one side of the depilated skin of the back 0.9 % NaCl, 10 % or 14 % sucrose as well as distilled water In parallel groups the same solutions were injected, containing 40 i.u. of hyaluronidase per dose (penetrase ®). 15 minutes later the animals were stunned by a blow on the neck, blood was withdrawn from the right side of the neck, and the mice killed by decapitation and bleeding. Two symmetrical areas, 5.5 cm² were marked on the skin of the back, one of the areas containing the injection zone. The cutis and the subcutaneous tissue was excised according to the method of SECHER-HANSEN,

*) A preliminary report of this investigation was given at the XII Scandinavian Congress of Physiology Turku, Finland, 1966.

LANGGÅRD & SCHOU (1967 a). The pieces of skin were hydrolyzed in 5 ml of 2N-KOH and the total radioactivity was determined in a well-counter (Isotope Developments Ltd.). Similarly the radioactivity of 50 μl of plasma from the same animal was determined. The radioactivity of each piece of skin was expressed in equivalents of the radioactivity of 1 μl plasma from the same animal. The difference in radioactivity expressed in this way between the injected and the non-injected piece of skin thus indicates the size of the inflammatory oedema in mg "plasma" (assuming that the composition of the oedema fluid at the time of observation corresponds to that of the plasma (cf. HVIDBERG, LANGGÅRD, SCHOU & SZPORNY 1964).

In other experiments different volumes were injected 0 μl (i.e. introduction of the hypodermic needle alone) 10, 40 or 80 μl of 0.9 NaCl, and the difference between the radioactivity of the injected and the non-injected pieces of skin was determined as described above.

Five minutes, 15 minutes and 2½ hours after injection of 80 μl of 0.9 % NaCl, 1.2 % NaCl, 6 %, 10 % or 14 % sucrose the right side, the difference between the total histamine content of the two pieces of skin was determined (cf. SECHER-HANSEN, LANGGÅRD & JANSEN 1967). In the same manner the difference in the total content of serotonin was determined after injections of 0.9 % NaCl and 10 % sucrose (cf JANSEN 1967).

Results

The accumulation of circulating ^{131}I human serum albumin in injected and non-injected pieces of skin, 15 minutes after the subcutaneous injections is shown in table 1 In all cases a significant accumulation was found on the side injected. Expressed in equivalents of 1 μl (= 1 mg) of plasma, the inflammatory oedema fluid varied from 6 to 14 mg (table 2). It appears

Table 1

Accumulation of circulating ^{131}I-human serum albumin in defined pieces of skin (1.5 cm²) expressed in equivalents of 1 μl plasma, 15 minutes after subcutaneous injection of 80 μl of different solutions. The radioactivity of symmetrical uninjected pieces of skin are also given. The figures are mean values ± standard error of the mean.

		0.9 % NaCl	10 % Sucrose	14 % Sucrose	H_2O	Uninjected sides
Untreated	Without Hyaluronidase	16 ± 1.8 n = 9	17 ± 2.3 n = 9	17 ± 1.1 n = 7	20 ± 1.5 n = 7	7 ± 0.3 n = 30
	With Hyaluronidase	17 ± 2.1 n = 8	20 ± 2.9 n = 9	18 ± 1.7 n = 7	21 ± [illegible]0 n = 6	
Oestradiol treated	Without Hyaluronidase	14 ± 0.8 n = 13	17 ± 2.1 n = 7		21 ± 2.5 n = 5	8 ± 0.9 n = 9
	With Hyaluronidase	17 ± 2.3 n = 8	17 ± 1.2 n = 8			

Table 2

Local uptake of circulating [131] I-human serum albumin 15 minutes after subcutaneous injection of various solutions, expressed in equivalents of 1 µl plasma. Since a dynamic equilibrium between the plasma and the inflammatory oedema fluid is present at the time of observation the figures represents the amount of oedema fluid in µl.

		0.9% N Cl	10% Sucrose	14% Sucrose	H_2O
Untreated	Without Hyaluronidase	9	10	10	13
	With Hyaluronidase	10	13	11	14
Oestradiol treated	Without Hyaluronidase	6	9	–	13
	With Hyaluronidase	9	9		–

from tables 1 and 2 that there is no significant difference between the size of the oedema after injection of 0 9 / NaCl, 10 / sucrose, 14 / sucrose or water Nor is there any significant difference in the extent of the oedema in normal animals and in animals pretreated with oestradiol. Similarly the addition of hyaluronidase to the solutions injected does not significantly alter the size of the oedema.

Fig. 1 shows graphically that by increasing the volume injected an increased accumulation of radioactivity is found.

Fig 2 shows that the subcutaneous injections do not at any time during the observed period produce any measurable alterations of the total histamine content of the injected pieces of skin, even when the solutions contained hyaluronidase. That the total content of serotonin in the tissue is also not altered, is apparent from fig. 3

Discussion

It has been acknowledged for many years that a subcutaneous injection represents a trauma of the tissue and therefore causes a local aseptic inflammation (BÁRÁNY 1932). It has further been assumed that the inflammatory reaction influences the course of the absorption process (FRANKE, BOATMAN, GEORGE & MOSES 1950). It has been maintained that injection of solutions of different composition (MADISON & CHRISTIAN 1950) and tonicity (WEHR, LEMMER & ELMAN 1950) affect the absorption

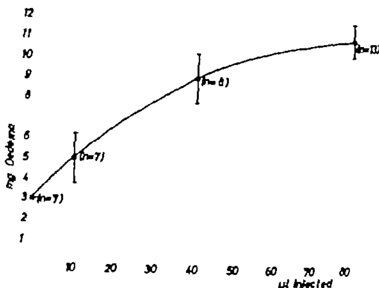


Fig. 1 Accumulation of circulating ^{131}I human serum albumin 15 minutes after subcutaneous introduction of the hypodermic needle only or after injection of 10, 40 or 80 μl of 0.9% NaCl into the right piece of two symmetrical pieces (5.5 cm² each) of depilated skin of the back of mice. The figures are mean values calculated as the difference between the total radioactivity expressed in equivalents of 1 μl plasma of the injected and the un-injected side. The standard error of the mean is given by vertical lines. n = number f animals.

rate in different ways. This might possibly be due to the extent of the inflammatory reaction being different after the injection of different solutions The present investigation confirms that subcutaneous injections cause a local aseptic inflammation (tables 1 and 2), but reveal that the extent of the oedema formation is largely independent of the composition of the solutions injected with regard to physiological and non-physiological substances, as well as in their tonicity (table 2). The latter is stressed by BÁRÁNY (1932) as being essential for the extent of the inflammation.

It has been shown in previous studies (SECHER HANSEN LANGGÅRD & SCHOU 1967a & b) that the state of the ground substance is essential for the subcutaneous absorption. This effect does not seem to be exerted through differences in the extent of the inflammation, the oedema formation being the same in (1) normal skin, in (2) skin in which the hyaluronic acid of the ground substance is depolymerized by means of hyaluronidase, and in (3) skin where the quantity of the ground substance is increased and the hyalyronic acid polymerized (COOPER & SCHMIDT 1957 HVIDBERG & JENSEN 1959 HVIDBERG, LANGGÅRD & SZPORNY 1963). Further this

5min. 15min. 150min.

Histamin µg / 5.5 cm² of injected skin

0.9% NaCl

1.2% NaCl

6% Sucrose

10% Sucrose

14% Sucrose

Without hyaluronidase

With hyaluronidase

Fig. 2. The difference between the total content of histamine in the injected and the uninjected pieces of skin (5.5 cm²) 5, 15 or 150 minutes (2½ h) after subcutaneous injections of 80 µl of various solutions with or without 40 I.u. of hyaluronidase per dose. The injection was given into the right piece of the two symmetrical pieces of skin of the back of living mice

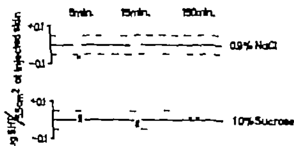


Fig. 3. The difference between the total content of serotonin in the injected and the uninjected piece of skin (5.5 cm²) 5, 15 or 150 minutes (2½ h) after subcutaneous injections of 80 µl of 0.9% NaCl or 10% sucrose. The injection was given into the right piece of the two symmetrical pieces of skin of the back of living mice.

investigation does not confirm the assumption that hyaluronidase, as well as being a spreading factor in the connective tissue, also increases the capillary permeability by a depolymerization of the ground substance in the capillary wall (ELSTER, FREEMAN & DORFMAN 1949 KELEMEN, IVANYI & MAJOROS 1951 SZABÓ & MAGYAR 1958)

The quantity of the volume injected subcutaneously however seems important in determining the degree of trauma, the extent of the inflammatory oedema produced increasing with increasing volume (fig. 1). The injection pressure presumably also plays a role (cf BARKE 1943)

Biogenic amines like histamine and serotonin are likely to be released by the trauma, and by means of a vasodilatation, to participate in the production of oedema (PARRAT & WEST 1956 WEST 1957 SPECTOR & WILLOUGHBY 1957 SCHOU 1961 WEIS 1963 NORN 1965 ZACHARIAE 1965) A reduced content of histamine and serotonin has been found in oedematous tissue However comparative investigations between normal and oedematous tissues involve difficult calculatory problems (LANGGÅRD & SECHER HANSEN 1967) In this work it was possible to compare the total content of histamine and serotonin in a defined piece of skin which was the site of an inflammatory reaction, with the content in an identical but non inflamed piece of skin. Expressed in this manner no alteration could be demonstrated in the total content of histamine or serotonin after subcutaneous injections of the solutions studied, nor when they contained hyaluronidase (fig. 2 and 3)

Summary

Subcutaneous injections represent a tissue trauma and will therefore produce a local aseptic inflammation. The extent of this inflammation is proportional to the volume injected. The extent of the inflammation was independent of the composition and the tonicity of the solutions injected. The state of the ground substance was found to be without significance in the oedema formation. Addition of hyaluronidase to the solutions injected did not increase capillary permeability The inflammatory reaction caused by subcutaneous injections did not result in any measurable decrease in the total content of histamine or serotonin in the tissue.

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Mrs. Vivi Halling and Mrs. Lone Jacobsen gave perfect technical assistance. The authors wish to thank The Danish Atomic Energy Commission for putting the well-counter at our disposal.

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Excretion of Noradrenaline, Adrenaline and Vanilmandelic Acid in Patients with Cerebro-vascular Disorders during Prenylamine Treatment

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Experimental studies have shown that prenylamine causes a decrease in the amount of noradrenaline in the heart and brain (SCHÖNE & LINDNER 1960 & 1962 JUORIO & VOGT 1965) as well as a release of adrenaline and noradrenaline from the adrenal medullary granules (SCHÖNE & LINDNER 1962 CARLSSON *et al* 1963 EULER *et al* 1964) from the perfused adrenal gland (JUORIO & VOGT 1965) prenylamine also causes a release of nor adrenaline from the splenic nerve granules (EULER *et al* 1964) Small concentrations of prenylamine also prevent the uptake of adrenaline and noradrenaline and decrease their spontaneous release (CARLSSON *et al* 1963 EULER *et al* 1963 GROBECKER *et al.* 1965 MACKENNA 1965 LUNDBORG 1966)

In patients with angina pectoris the urinary excretion of total catecholamines, particularly that of adrenaline and vanilmandelic acid (VMA), was increased in some patients during the first five days of prenylamine treatment (KUSCHKE *et al* 1964), while RAUEN & ALBERT (1964) observed a significant decrease in the VMA excretion in 7 out of 10 healthy subjects after prenylamine treatment for 14–16 days.

The purpose of the present study was to determine the effect of prenylamine treatment on the urinary excretion of noradrenaline, adrenaline and their main metabolite VMA in patients with cerebro-vascular disorders during 1–3 and 11–13 days respectively from the beginning of the treatment.

Material and Methods

Our material consisted of 29 patients (16 female and 13 male) of the Neurological Clinic, who all suffered from cerebro-vascular disorders. The mean age was 52 years. In none of the patients were any functional disturbances observed in the liver or kidneys.

In order to improve the blood circulation of the brain the patients were treated with prenylamine (2-(3,3-Diphenylpropylamine)-1-phenylpropane = segontin ®, Hoechst) 120 mg daily orally. During 3 consecutive days the patients' 24-hour urine was collected into hydrochloric acid 1) immediately before the treatment, 2) during the first three days of treatment and 3) on the 11th–13th days of treatment. The patients were hospitalized for a mean period of 7 days (range 1–38 days) before prenylamine treatment and also during the 13 days of treatment. They received no other drugs during this time. Interferences from dietary substances in the VMA determination was not observed by the method of (PISANO et al. 1962) used for the determination of VMA. Stress situations such as surgical operations (PEKKARINEN et al. 1963 & 1965a), renal (PEKKARINEN & IISALO 1965a) and cardiac (PEKKARINEN & IISALO 1965b) insufficiency and treatment with monoamine oxidase inhibitors (PEKKARINEN et al. 1965b) which influence the urinary excretion of VMA, did not occur in our patients.

The noradrenaline and adrenaline were determined fluorimetrically by PEKKARINEN's (1964) modification of v. EULER's & FLODING's (1956) potassium ferricyanide method and according to PRICE & PRICE (1957) on the basis of the wavelength differentiation of adrenaline and noradrenaline. The vanilmandelic acid (VMA, 3-methoxy-4-hydroxymandelic acid) was determined by a micromodification (PEKKARINEN & HAKULINEN, unpublished) of the method of PISANO et al. (1962).

Results

1) *Excretion of total catecholamines*

Before the prenylamine treatment the mean excretion of total catecholamines was 68.4 ± 5.7 µg/24 hours (Table 1). During the prenylamine treatment no significant changes took place: the excretion on the 3 first days of treatment was 66.0 ± 5.3 µg/24 hours and on the 11th–13th days 58.8 ± 4.8 µg/24 hours (differences, $P > 0.05$). The mean excretion of the total catecholamines during three consecutive days also did not differ very much from each other. Thus on the basis of the daily variation of the excretion no clear changes were observed in the individual patients.

2) *Excretion of noradrenaline*

The mean excretion during the control period was 61.9 ± 5.0 µg/24 hours (Table 1). During days 1–3 and 11–13 of treatment the excretion was almost the same as on the control days, i.e. 58.3 ± 5.0 µg/24 hours and 52.6 ± 6.8 µg/24 hours respectively (differences, $P > 0.05$). The mean noradrenaline excretion on three successive days did not deviate very much, nor could any clear changes be observed in the individual patients.

Table 1

Excretion of total catecholamines (CA, μg/24 hours), noradrenaline (NA, μg/24 hours), adrenaline (A, μg/24 hours) and vanillmandelic acid (VMA, mg/24 hours) in patients with cerebro-vascular disorders before and during prenylamine treatment (120 mg daily). Mean ± S.E.M

	Pretreatment control (days)				Time f treatment (days)							
	1		3	Comb. Mean	1	2	3	Comb. Mean	11	12	13	Comb. Mean
Total CA (μg/24 hours)												
Mean	64.5	63.9	76.9	68.4	65.3	66.9	66.3	66.0	58.4	60.9	61.7	58.8
S.E.M	±9.6	±7.1	±12.5	±5.7	±7.0	±9.3	±12.7	±5.6	±8.7	±8.5	±8.5	±4.8
n	(23)	(23)	(23)	(23)	(23)	(22)	(15)	(23)	(20)	(21)	(18)	(21)
P)								>0.05				>0.05
NA (μg/24 hours)												
Mean	59.4	57.6	68.6	61.9	57.4	58.3	59.9	58.3	48.4	54.1	55.4	[illegible]
S.E.M	±8.9	±6.4	±10.3	±5.0	±6.4	±8.7	±12.2	±5.0	±8.0	±8.7	±8.6	±6.8
n	(23)	(23)	(23)	(23)	(23)	(22)	(15)	(23)	(20)	(21)	(18)	(21)
P)								>0.05				>0.05
A (μg/24 hours)												
Mean	5.1	6.3	8.2	6.5	7.9	8.6	6.5	7.7	6.0	6.8	5.9	6.2
S.E.M	±1.4	±1.8	±3.1	±1.3	±2.3	±4.9	±2.1	±1.5	±1.9	±1.7	±1.5	±1.0
n	(23)	(23)	(23)	(23)	(23)	(22)	(15)	(23)	(20)	(21)	(18)	(21)
P)								>0.05				>0.05
VMA (mg/24 hours)												
Mean	4.2	4.4	4.9	4.5	4.4	4.0	4.2	4.2	3.8	3.3	3.8	3.6
S.E.M	±0.33	±0.30	±0.33	±0.19	±0.27	±0.28	±0.38	±0.18	±0.29	±0.20	±0.34	±0.15
n	(29)	(29)	(29)	(29)	(29)	(27)	(22)	(29)	(18)	(18)	(13)	(18)
P)								>0.05				<0.05

) P compared with the corresponding controls.

3) *Excretion of adrenaline*

Before treatment the mean excretion of adrenaline was 6.5 ± 1.3 µg/24 hours (Table 1) During treatment no significant changes took place (7 7 ± 1.5 µg/24 hours and 6.2 ± 1 0 µg/24 hours respectively differences, P > 0 05) The mean adrenaline excretion during the three successive days did not deviate very much. In the individual patients no clear increase or decrease in the excretion was observed.

4) *Excretion of VMA*

The mean excretion of VMA during the control days was 4.5 ± 0.19 mg/24 hours (Table 1) At the beginning of treatment the excretion was 4.2 ± 0 18 mg/24 hours and on 11–13 days of treatment almost at the same level, i.e. 3 6 ± 0 15 mg/24 hours, though this decrease as compared with the control value was statistically significant ($P < 0.05$). The mean VMA excretion during three successive days between individual patients are of the same order

Discussion

The effect of prenylamine on the metabolism of adrenaline and noradrenaline is only partly known. It has been shown that it does not inhibit dopa-decarboxylase but it certainly prevents the activity of monoamine oxidase *in vitro* although not clearly *in vivo* (Schöne & Lindner 1960 & 1962 Wohlrab 1961) As prenylamine causes release of catecholamines and inhibits their uptake (Schöne & Lindner 1962 Carlsson *et al* 1963 Euler *et al* 1964) it is justifiable to suggest that prenylamine influences adrenaline and noradrenaline in the same way as reserpine, although its effect is weaker (Schöne & Lindner 1962 Euler *et al* 1964 Juorio & Vogt 1965 Lundborg 1966)

The results of the present study show that prenylamine in a daily dose of 120 mg had no significant effect on the urinary excretion of noradrenaline and adrenaline for two periods of treatment, i.e. days 1–3 and 11 13 from the beginning of treatment, in 29 patients with cerebro-vascular disorders. This result is not in agreement with the observations of Kuschke *et al* (1964) in some patients with angina pectoris. In these, the urinary excretion, especially that of adrenaline was increased, from which it has been assumed, that the cells of the adrenal medulla would be particularly sensitive to the effect of prenylamine. However a statistically significant, although small decrease in the VMA excretion occurred on the 11th–14th

days of treatment, as has also been observed in healthy subjects (RAUEN & AEBERT 1964). During reserpine treatment there is also a decrease in the VMA excretion in the patients (RANDRUP *et al* 1963, ALLEGRANZA *et al* 1965). Reserpine also decreases the excretion of noradrenaline and adrenaline into the urine (GADDUM *et al* 1958, CARLSSON *et al* 1959), although it has been found that the adrenaline excretion increases in patients with toxemia of pregnancy during bed rest in hospital (CASTRÉN 1963). Reserpine has a much more marked effect than prenylamine on the content and release of adrenaline and noradrenaline in tissues.

Summary

The urinary excretion of noradrenaline, adrenaline and vanillmandelic acid (VMA) was studied before and during days 1–3 and 11–13 of prenylamine treatment, 120 mg daily in 29 patients with cerebro-vascular disorders.

Before this treatment the mean excretion of total catecholamines was 68.5 ± 5.7 μg/24 hours, that of noradrenaline 61.9 ± 5.0 μg/24 hours, of adrenaline 6.5 ± 1.3 μg/24 hours and of VMA 4.5 ± 0.19 mg/24 hours. During prenylamine treatment there was no significant change in the total excretion of noradrenaline or adrenaline. On the other hand the excretion of VMA on days 11–13 of treatment (3.6 ± 0.15 mg/24 hours) decreased slightly but significantly ($P < 0.05$) from the control value.

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Inhibition of 17β-Oestradiol Uptake in Mouse Uterus by Doisynolic and Allenoic Acid Derivatives An *in Vitro* Differentiation between True Oestrogens and Pro-Oestrogens

By

Lars Terenius

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Besides the oestrogen which occurs naturally in the animal kingdom many synthetic compounds with oestrogenic activity are known. The synthetic oestrogens are often structurally different from those occurring naturally (cf GRUNDY 1957) and is is questionable if all these compounds act by the same mechanism. It is now well established that a natural oestrogen, 17β-oestradiol, accumulates in "target organs" such as the uterus and vagina of rodents and is retained there (JENSEN & JACOBSON 1962 STONE *et al* 1963 TERENIUS 1965) The synthetic oestrogen *meso*-hexoestrol is taken up by the reproductive organs of sheep and goats (GLASCOCK & HOEKSTRA 1959) *meso*-Hexoestrol is also taken up by the uterus and vagina of immature mice in much the same manner as 17β-oestradiol (TERENIUS 1966b).

In a previous investigation (TERENIUS 1966a) it was found that various synthetic oestrogens blocked the uptake of 17β-oestradiol by the uterus and vagina in immature mice to an extent which was roughly proportional to their oestrogenic activities Of the investigated compounds, the allenoic acid derivative, vallestril ® (methallenostril), however was a poor uptake inhibitor in relation to its oestrogenicity It was considered possible that vallestril had to be metabolized in order to become active. Vallestril has a methyl-ether group and as a first approach demethylation of vallestril and of the structurally related oestrogen fenocyclin ® (a doisynolic acid derivative) was undertaken. The effects of these demethylated substances and the parent compounds on the uptake of tritium-labelled 17β-oestradiol by the uterus and diaphragm in immature mice was then investigated

The experiments were largely carried out *in vitro* to diminish the possibility of further chemical transformation. Structural specificity for the inhibition of 17β-oestradiol uptake by the uterus can be demonstrated *in vitro* (cf TERENIUS 1966c)

Materials and Methods

17β-oestradiol (oestra-1,3,5(10)-triene-3 17β-diol) labelled with tritium at the 6 and 7 positions was purchased f om New England Nuclear Corporation. Its specific activity was 140 μc/μg. On chromatography on silica gel thin layer plates in chloroform/acetic acid (85 15) it had a radiochemical purity of at least 98 %.

Fenocyclin Ⓡ (DL 7-methyl-bisdehydrodoisynolic acid DL-1-ethyl-1,2,3,4-tetrahydro-7 methoxy-2-methyl-2-phenanthrenecarboxylic acid) and vallestril Ⓡ (methallenestril = DL 3-(6-methoxy-2-naphtyl)-2,2-dimethylpentanoic acid) were commercial preparations and melted at 225–228° (228–230°*) and 141–142.5 (132) respectively They were demethylated by hydrobromic acid according to conventional methods. The fenocyclin derivative (DL 1-ethyl-1,2,3 4-tetrahydro-7-hydroxy-2-methyl-2-phenanthrenecarboxylic acid) melted at 197–200° (204°*) and the vallestril derivative (DL-α,α-dimethyl-β-ethyl-allenolic acid DL 3-(6-hydroxy-2-naphtyl)-2,2-dimethylpentanoic acid) at 175–176° (170–171). They were homogeneous in several thin layer chromatographic systems (cf. table I).

The chemical structures of the investigated compounds are given in fig. 1 The formulae indicate the generic relationships between the compounds. Encircled carbons are asymmetric.

16 to 18 days old mice of the N M R.I. strain weighing 9 t 11 g were used. For *in vitro* experiments they were killed by blow on the head and their uteri and diaphragms immediately cut out. The uterus was divided into two identical parts at the cervix nd the diaphragm divided int strips weighing bout 3–4 mg. A piece of tissue was then taken from each of two animals and placed in each incubation flask i.e. two half-uteri and two strips of diaphragm per flask. The incubation flasks contained 3 ml Krebs-Ringer phosphate buffer pH 7.4, prepared from reagent chemicals and re-distilled water as described by COHEN (1957). Bovine albumin (Cohn fraction V Sigma B grade) was added at a final concentration of

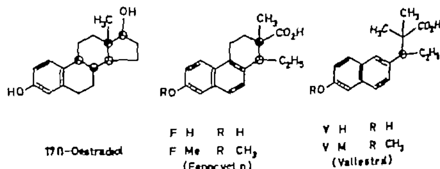


Fig 1 Formulae of 17β-oestradiol and derivatives of fenocyclin Ⓡ and vallestril Ⓡ (= methallenestril).

*) Values within brackets from HOGG & KORMAN (1956).

2% (w/v). Appropriate aliquots of alcoholic stock solutions of the test compounds were taken to dryness and redissolved in the buffer. The incubation flasks were shaken in a Warburg apparatus. In some experiments (table 2) the tissues were pre-incubated in flasks containing the non-radioactive compound and then transferred to other flasks containing radioactive 17β-oestradiol. The tissues were incubated in air and after incubation the tissues were gently blotted between two filter papers and weighed wet on a torsion balance. Less than 5% of the medium activity was recovered in the tissues. The radioactivity accumulated *in vitro* by the uterus on incubation with 17β-oestradiol-^{3}H has been shown to consist mainly of the unchanged compound (unpubl.). The *in vitro* experiments were carried out as previously described (Terenius 1965).

The tissues were solubilized in scintillation vials by 1 ml Hyamine /toluene (1 3) in a shaking incubator at 70° 5 ml of scintillation solution (0.5% PPO 0.03% dimethyl-POPOP in A.R. toluene) was added and the samples were measured in a Tri-Carb liquid scintillation spectrometer model 3324. Most samples were measured with about 25% counting efficiency and at least 10000 counts were recorded. Known amounts of tritium activity were then added, the samples recounted and the activities calculated as disintegrations per minute and unit wet weight (DPM/mg).

Results

Chromatographic behaviour of the oestrogens

The oestrogens investigated are chemically different. Chromatography was carried out in order to characterize their hydrophilic-lipophilic properties. The chromatographic mobility of the carboxylic acids was strongly pH-dependent while the mobility of 17β-oestradiol was not (table 1).

Tissue incubation with mixtures of labelled 17β-oestradiol and synthetic oestrogen

Various amounts of the methyl-ethers fenocyclin ® (F-Me) and vallestril ® (V Me) were incubated together with 0 0015 μg tritium-labelled 17β-oestradiol in 3 ml of medium and the uptake of radioactivity by the uterus and the diaphragm measured. There was no marked effect on the uptake in the dose range studied, except possibly for a slight inhibition by 10 μg of F Me which was not significant (fig. 2) On the other hand, the demethylated analogues F-H and V H inhibited the uterine uptake of 17β-oestradiol (fig. 3) The preferential uterine uptake was depressed almost to the diaphragm level at a concentration of 10 μg per 3 ml of medium. The diaphragm content remained constant over the whole dose range.

) Packard Co.

Table 1

The chromatographic behaviour of oestrogenic carboxylic acids compared with that of 17β-oestradiol About 25 μg of each compound was run on silica gel thin layer plates (Merck Kieselgel H). The solvent front was allowed to migrate bout 11 cm. The plates were evaluated by charring with 5% $KMnO_4$ in concentrated H_2SO_4. The table gives R_F values.

Compound	Solvent system		
	Chloroform/ Acetic acid (85 15)	Benzene/ Methanol (60 10)	Chloroform/ Diethylamine(80:20)
17β-Oestradiol	0.5	0.30	0.55
F-H	0 91	0.30	0.05
F Me	0 67	0.16	0.02
V H	0 91	0.34	0 05
V Me	0 65	0.20	0.03

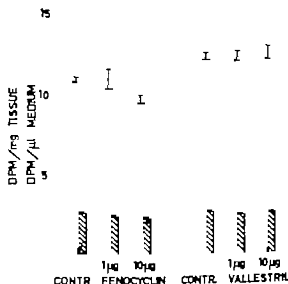


Fig 2. Effect of fenocyclin (F Me) and vallestril (V M methallenestril) on the uptake of 17β-oestradiol by the uterus and diaphragm / /ro. There was 0 0015 μg 17β-oestradiol-³H and the indicated amounts f the test compounds in 3 ml medium. Incubations lasted for 1 hour at 37° Tissues were taken from 4 nimals per group Concentration ratios on the ordinate are calculated $\frac{\text{DPM/mg wet tissue}}{\text{DPM/μl medium}}$ Mean values ± s.e.m. (standard error of the mean) are given. Open columns represent the uterus and the cross-hatched columns the diaphragm.

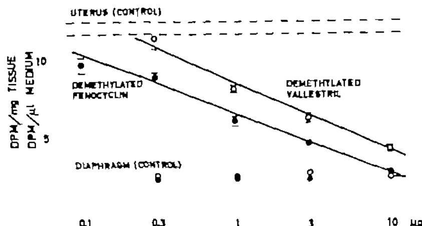


Fig. 3. Effect of the demethylated analogues of fenocyclin (F H) and vallestril (V H) on the uptake of 17β-oestradiol by the uterus and diaphragm in vitro. There was 0.0015 µg 17β-oestradiol-³H and the indicated amounts of inhibitor per 3 ml medium. Tissues were taken from 4 animals per group. Mean values of concentration ratios are given; s.e.m. are depicted for the uterus whilst for the diaphragm they were ±0.01 or less.

Pre incubation experiments

It has been found that 17β-oestradiol and *meso*-hexoestrol are retained by the mouse uterus *in vitro* while the diaphragm rapidly loses its content (unpubl.). One might question whether compounds F H and V H are also specifically retained by the uterus. These compounds labelled with tritium to a high specific activity were not available to the author but it was possible, however, to analyse the problem by indirect means. Provided that e.g. F H is retained by the uterus, pre incubation with this compound alone would be expected to inhibit the uptake of radioactivity when the tissues were subsequently incubated with labelled 17β-oestradiol. Model experiments with 17β-oestradiol and *meso*-hexoestrol were carried out. The results in table 2 show that F H, *meso*-hexoestrol and "cold" 17β-oestradiol were about as effective as inhibitors of the uptake of labelled 17β-oestradiol when used in the pre-incubation, as when used in the ordinary simultaneous incubation. It is therefore highly probable that F H is also retained by the uterus. This result also indicates that the inhibition of 17β-oestradiol uptake by F H is not due to interactions in the medium but to interactions actually within the uterine tissue.

In vivo experiments

A good correlation between the uptake of hexoestrol isomers by the

Table 2

Effect of simultaneous addition of or pre-incubation with, 17β-oestradiol, *meso*-hexoestrol and compound F H on the *uptake of 17β-oestradiol-³H by the uterus and diaphragm.* The "simultaneous" method involved incubation in a mixture of the non-radioactive compound and the radioactivity for 1 hour at 37° Otherwise, the tissues were pre-incubated with the non-radioactive compound for 1 hour at 37° followed by an incubation with 17β-oestradiol-³H for 1 hour at 37 Tissues in the control group were only incubated with radioactive 17β-oestradiol for 1 hour at 37 Concentration ratios are mean values ± s.e.m., tissues were taken from 4 animals per group.

Compound, amount	Treatment	Content of radioactivity: Uterus/Medium	Diaphragm/Medium	Uterus/Diaphragm
None	control	10.3 ± 0 6	2.0 ± 0.07	5.2
17β-Oestradiol, 0.1 µg	simultaneous	1.8 ± 0.1	1.6 ± 0.01	1 1
17β-Oestradiol, 0.1 µg	pre-incubation	2.4 ± 0.4	1.9 ± 0.1	1.3
meso-Hexoestrol, 0.3 µg	simultaneous	3 1 ± 0.03	2.1 ± 0.06	1.5
meso-Hexoestrol, 0.3 µg	pre-incubation	*3 7 ± 0 1*	*2.1 ± 0.1*	1.8
F H 3 µg	simultaneous	4.4 ± 0.[illegible]	1 6 ± 0.01	2.7
F H 3 µg	pre-incubation	5.1 ± 0.4	1 7 ± 0.07	3 0

uterus *in vivo* and *in vitro* has already been demonstrated earlier (TERENIUS 1966c). The following experiments show that the free phenol F H which inhibited the uterine uptake of 17β-oestradiol *in vitro* also inhibits this uptake *in vivo* Compound F Me was inactive at the same concentrations (fig. 4)

Discussion

The accumulation of oestrogens by target organs is most likely an important factor affecting their potency The author has demonstrated elsewhere (TERENIUS 1966a) a reasonable correlation between the capacity of certain oestrogens to inhibit 17β-oestradiol uptake *in vivo* and their oestrogenicity

The effect of some synthetic oestrogens on the specific uterine uptake of labelled 17β-oestradiol *in vitro* is summarized in table 3 The ratio between activity for uptake inhibition and uterotrophic activity were similar for *meso*-hexoestrol and compounds F H and V H On the other hand, fenocyclin (F Me) and vallestril (V Me) were not inhibitory at doses which were high in relation to their oestrogenicity It is therefore most probable that the latter compounds, in order to reach the specific sites and produce their biological effects, have to be demethylated to the

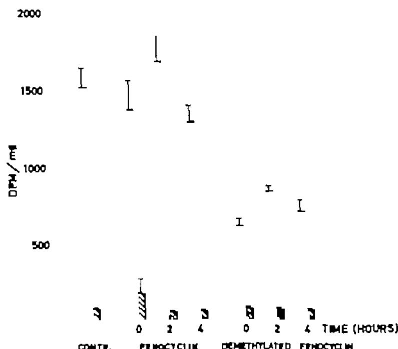


Fig. 4. Effect of fenocyclin (F-Me) and its demethylated analogue (F H) on the uptake of 17β-oestradiol by the uterus and diaphragm *in vivo*. All injections were made into the orbital plexus. The radioactivity was injected into one plexus and the inhibitor into the other. Each animal received 0.02 μg 17β-oestradiol-^{3}H in saline and the experimental groups received in addition 10 μg inhibitor dissolved in 10 % propylene glycol in saline. The inhibitors were injected simultaneously or at various times before the radioactivity. There were 4 to 5 animals in each group. Radioactivity is expressed as DPM per mg wet tissue. Open columns represent the uterus and cross-hatched columns the diaphragm. Vertical bars denote s.e.m.

corresponding free phenols. Using the terminology of Emmens and collaborators (e.g. Emmens 1941) Fe Me and V Me thus appear to be pro-oestrogens. The same conclusion can be drawn from findings by other authors. Chedid & Horeau (1951) working with immature rats, actually found that V Me was inactive in the Astwood 6 hour test (Astwood 1938) while the demethylated analogue V H was highly active. However V Me produced a slow increase in uterine wet weight giving a maximum at 48 hours after a single dose, and at that time the wet weight equalled that induced by similar treatment with V H. Similarly it is shown in table 3 that the methyl-ethers F Me and V Me are about equally uterotrophic as the free phenols F H and V H if given sufficient time to act, i.e. presumably enough time to be demethylated (see also Miescher 1948).

Table 3

The relation between the uterotrophic activity and the uptake inhibitory activity *in vitro* of some oestrogens. Uterotrophic doses* in column II were given daily for three days. For *in vitro* experiments the incubation flasks contained 0.0015 µg 17β-oestradiol-^3H and various quantities of inhibitors in 3 ml medium. In column III, doses of inhibitors giving a 70 % inhibition of the specific *in vitro* uterine accumulation (total uterine concentration of radioactivity minus diaphragm concentration of radioactivity) are given. The ratio R in column IV should be interpreted $R = \frac{\text{Inhibitor amount (column III)}}{\text{Uterotrophic dose (column II)}}$

I	II	III	IV
Compound	Uterotrophic dose (giving 50 % of maximum response), µg	Inhibitory amount *in vitro* (giving 70 % inhibition of specific uterine uptake), µg	R
meso-Hexoestrol	0.015	0.3	20
F H	0.10	1.7	17
F Me	0.12	>10	>80
V H	0.07	5.0	7
V Me	0.15	>10	>70

Incidentally etherification of the phenolic group of oestrogenic steroids results in a marked loss of biological activity. Various alkyl-ethers of oestrone have about 1/50 of the activity of the parent compounds (ERCOLI, GALLETTI & FALCONI 1962) on subcutaneous administration daily for 3 days to immature mice. Fenocyclin (F-Me) and vallestril (V Me) thus seem to be de-alkylated *in vivo* faster than ethers of oestrone. In the chicken oviduct test for oestrogens, the situation is different. In low doses, F Me has been found to be equipotent with 17β-oestradiol-3-benzoate but the maximum response reached with F Me is much lower (DORFMAN & DORFMAN 1948). This finding has been confirmed in our laboratory and it was also found that compound F H gives a maximum oviduct response which approaches that of 17β-oestradiol 3-benzoate. It is therefore probable that in the chicken demethylation of F-Me is a rate-limiting step.

The findings reported above, support the hypothesis of CLARK (1950), GRUNDY (1957) and others that a molecular requirement for oestrogenic activity is *two* separate hydrophilic groups. The distance between the hydrophilic groups of F H and of 17β-oestradiol are also similar when measured on molecular models in their most extended conformations. Compound V H is less rigid, but as is evident from the formula chart it

can be superimposed on compound F-H. Furthermore, JENSEN & JACOBSON (1962) found that 17β-oestradiol, but not the mono-hydroxy compound oestrone, is taken up by the uterus of immature rat and STONE & BAGGETT (1965) made a similar observation with regard to the vagina of the ovariectomized mouse.

The compounds F-H, F Me, V H and V Me are substituted carboxylic acids which in general have a pKa of about 5. This means that at physiological pH most of the compound is ionized. The solubility and polarity are therefore different from the phenolic oestrogens as was demonstrated by thin layer chromatography on silica gel (table 1). The silica gel is faintly acid. In acid or neutral solvents, the carboxylic acids have mobilities not very different from 17β-oestradiol. However, in the faintly alkaline system with diethylamine, where 17β-oestradiol has unchanged mobility the carboxylic acids are ionized and hardly move at all. Thus, the carboxylic acids can be expected to behave differently from 17β-oestradiol with regards to solubility e.g. in the lipids of the cell membranes. This also indicates that the interaction between the carboxylic acids and 17β-oestradiol for uptake in the uterus indeed involves specific sites.

Summary

The oestrogenic acids vallestril ® (methallenestril) and fenocyclin ® which are methyl-ethers did not inhibit the uptake of 17β-oestradiol *in vitro* by the immature mouse uterus. After demethylation however they were strongly inhibitory. It is suggested that the accumulation of these demethylated derivatives at the specific sites is responsible for the oestrogenic effects of the parent compounds.

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Lack of Effect of Administration of Fluoride on the Central Nervous System of Rats

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Although the effects of fluoride on dental caries, bone formation, enzyme activity and general health have been investigated extensively little information is available regarding its effect on the activity of the central nervous system (CNS) Among the few studies in the literature relevant to the CNS are those on cholinesterase (ChE) inhibition (MATTHES 1930 NACHMANSOHN 1939 DYRENG & LOE 1956), and on depression of electrical activity of the cerebral cortex of the "encephale isolé" cat (MORUZZI 1938). In rats, sodium fluoride was reported to potentiate the effects of certain centrally acting agents including pentobarbitone and diphenylhydantoin possibly by inhibiting ChE (LU *et al.* 1961 RICE & LU 1963)

Several investigators (RUSSELL 1956 WATSON 1959 LASAGNA & LATIES 1959 RUFFIN 1963) have suggested that toxic effects of drugs on the CNS may show up earlier in altered behavioural patterns than in more readily observable physiological changes. Thus, subtle changes in behaviour such as impaired memory or learning ability might occur at lower dosage levels than, for instance, changes in respiration and heart rate, gross motor inco-ordination or general depression of activity It was therefore considered desirable to investigate the effect of sodium fluoride on maze learning ability in rats, and to use pentobarbitone sleeping time as a comparative physiological measure.

Methods

45 male hooded rats were divided into three equal groups at the [illegible] of 42 days. Group 1 was given a standard laboratory diet) t which had been added 150 ppm of NaF (68 ppm of F^-), Group 2 received the same diet with 15 ppm of added N F (6.8 ppm of F^-) and Group 3 the control group, received the standard diet without any added fluoride. The concentrations of N F added to the diets were the same as those reported by LU *et al*

) Ground Master Fox Cubes (fluorine content 0.002 %), Maple Leaf Milling Co., Toronto, Ont.

(1961). The concentration of fluoride in drinking water was well below 1 ppm. Food and water were available *ad libitum*, except for some weeks before and during the maze experiment, when food was rationed. The daily intake of food was recorded and the animals were weighed at weekly intervals. All the rats were housed in individual cages and kept in an air conditioned room maintained at 72–78°F

Approximately 4 months after the beginning of fluoride treatment the rats were tested for differences in learning ability using an elevated multiple T-maze with 30 points of choice. Each arm of the maze, including the cul-de-sacs, was 1½ wide and 18" long, and there were 4 square platforms at both the starting and goal points. The whole maze was placed on stilts 24 above floor level.

The food of all rats was rationed daily two weeks before the learning test to reduce body weights to pproximately 80% of normal. This level of weight reduction generally produces optimal motivation f r running a maze for food reward, and all rats were kept at 80% of normal weight throughout the maze learning experiment. The reward used in this experiment consisted of wet mash at the goal.

The animals received five days f preliminary training on a single unit of the T-maze to adapt them to the experimental situation. Then each rat was given one trial per day for 9 days in the complete maze. The time taken to reach the goal from the starting point (latencies) and the number of wrong turns into blind alleys (errors) were recorded for each trial. On arriving at the goal the rats were allowed to feed for 10 seconds before being removed. If rat had not reached the goal in 15 minutes it was immediately returned to its home cage until the following day All rats received their daily rations of food in the home cages after the trials.

On completio of the maze learning experiment, 10 rats from each group were maintained on their respective diets with *ad libitum* feeding for further period of 3 months, during which time one rat in the control group died of respiratory disease. The remainder were submitted to a pentobarbitone induced (35 mg/kg of pentobarbitone sodium injected intraperitoneally) sleeping-time test, duri g which the time taken by the rats to regain the righting reflex was recorded.

Results

No significant differences were found in the mean weights of the animals in the three groups nor in their mean food intakes throughout the ex periment. Data on the actual fluoride intake were calculated from the daily food consumption per rat and the results are listed in table I The

Table 1

Daily fluorine intak in mg per rat.

Period	Group 1 (68 ppm F^-)	Group (4 8 ppm F^-)
Ad lib. feedi g. Mean for one month before rationing	1 37 0.025)	0.14 ± 0.003
Rationed feeding. Mean for 2 weeks before ad 2 weeks during maze experiment	0.67 ± 0.025	0.06 ± 0.003

) Standard error

Table 2.

Maze Learning Experiment – Medians and ranges of latencies*) and error scores) (15 rats/group).

Group	Trial 1		Trial 5		Trial 9 (final trial)	
	Latencies	Errors	Latencies	Errors	Latencies	Errors
1 (68 ppm F^-)	625 (335–900+)	14 (12 16)	105 (40–430)	2 (0–9)	78 (38–255)	0 (0–2)
2 (6.8 ppm F^-)	900+ (395–900+)	14 (7 19)	145 (45–900+)	3 (0–11)	93 (41–412)	1 (0–2)
3 (Control)	565 (245–900+)	14 (6–17)	130 (55–900+)	2 (0–7)	85 (37 265)	0 (0–1)

) Time in seconds.

*) Error scores of rats which did not complete the maze any one trial were computed in proportion to the number of choice points passed, e.g. 5 errors and 10 choice points passed = $\frac{5 \times 30}{10}$ = 15 errors.

fluorine present in the basal diet was derived from bone meal and as such was assumed to be largely unavailable (MACHLE & LARGENT 1943 JACKSON *et al* 1950 TAYLOR & GARDNER 1959)

The results of the maze learning experiment (table 2) generally showed higher median scores in Group 2 than in the other two groups, but the spread of the scores was wide with complete overlap in each group Since the distribution was markedly skewed, a non-parametric test was considered to be the most suitable for statistical analysis. The Mann Whitney U-test (SIEGEL 1956) showed no significant differences in the rate of learning (as indicated by error scores and latencies) at any stage of the experiment between any of the groups.

Table 3 presents data for the sleeping-time test. Since the individual values again did not appear to be normally distributed, with three of the rats being unaffected by the pentobarbitone injection and two sleeping for longer than 3 hours, the Mann-Whitney U test was again used to determine the extent of difference between the groups. No significant differences were found in the pentobarbitone sleeping times of the various groups.

Discussion

Our observation that small amounts of NaF failed to influence maze learning ability in rats suggests two possible explanations 1) that the

Table 3

Duration of pentobarbitone-induced sleep (time in minutes).

Group 1 (n = 10)	Group 2 (n = 10)	Group 3 (= 9)
(0)	22	(0)
40	43	(0)
44	52	19
54	53	60
79	63	103
81	79	112
89	85	115
99	87	149
115	144	(180+)
140	(180+)	
740	628	558
Median = 80	71	103

chronic administration of fluoride in low doses has no effect on the learning ability and memory of rats, or 2) that a maze learning task is not sufficiently sensitive to reveal the effects of small amounts of fluoride on the CNS

After completion of the present experiments, several reports of similar studies, which had produced conflicting results were found in the literature. When sodium fluoride was administered to rats in the drinking water impairment of conditioned reflex activity was found with as little as 1 ppm of fluorine (AKSYUK & BULYCHEV 1962) Similarly KNIZHNIKOV *et al.* (1963) observed depression of the EEG with 15 ppm of fluorine. On the other hand GABOVICH (1962) reported that up to 4 ppm of fluorine in the water produced no significant difference in performance on a combined maze and discrimination learning task. A direct comparison between the present work and that of the above mentioned investigators is difficult because of inadequate information on the behavioural methods used by them, and on the levels of water intake by their rats. The maximum concentration of fluoride used by us was much higher than those reported by other investigators, even if one takes into account the lower absorption rate from food than from water (WUTHIER & PHILLIPS 1959). Our maze learning experiment, however was only performed after 4 months of chronic fluoride administration, whereas the tests of the other investigators were performed after 6–9 months of fluoride administration.

Conditioned reflex methods are considered by most Soviet and some

Western toxicologists to be more sensitive than other toxicological tests (RUFFIN 1963) However GOLDBERG *et al* (1964) have recently suggested that the tests used at present for measuring behavioural responses are not always more sensitive than physiological methods. The observation of KNIZHNIKOV *et al.* (1963) that the EEG was affected by fluorine, although maze learning evidently is not, may thus indicate the relative insensitivity of the latter as a measure of behavioural toxicity

The previously reported prolongation of pentobarbitone-induced sleeping-time in fluoride treated animals (LU *et al.* 1961) was not confirmed in the present study This may be due to the use of male hooded rats instead of female albinos which were used in the experiment of LU *et al* (1961) It is known that female rats are more sensitive than males to barbiturates (BRODIE 1962) and to fluoride (MARCOVITCH & STANLEY 1938 DYBING & LOE 1956 RAMSEYER *et al* 1957 WUTHIER & PHILLIPS 1959), and, although no information is available, there may also be a difference in sensitivity between the two strains Thus, in our experience, a dose of 35 mg/kg i p of pentobarbitone was required to bring about the loss of righting reflex in most of the animals, whereas 20–25 mg/kg i.p was sufficient in the experiment of LU *et al* (1961) Lower sensitivity to fluoride in the hooded males may also account for the absence of effect on maze learning.

Summary

Three groups of 15 male hooded rats each were given a control diet alone or one supplemented with 15 and 150 ppm of NaF Four months later they were tested for speed of learning of a multiple T maze. The criteria used were running latencies and the number of errors. No significant differences were found between the three groups. Three months later 10 rats from each experimental group and 9 from the control group were submitted to a pentobarbitone-induced sleeping-time test. Fluoride had no effect on sleeping time.

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A Modification of the Rat Fundus Strip Method by Vane for Assay of 5-Hydroxytryptamine (Serotonin)

By

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(Received January 2, 1967)

The content of 5-hydroxytryptamine (5-HT) in biological fluids and tissue extracts is frequently determined spectrophotofluorometrically (BOWMAN GAULFIELD & UDENFRIEND 1955) or photofluorometrically after coupling with ninhydrin (VANABLE 1963). These methods are very sensitive and can be made fairly specific by application of suitable extraction and purification processes.

In cases where only few analyses are required, however it is still more convenient to use a biological method since the chemical methods involve the development of extraction and separation procedures. In laboratories where a spectrophotofluorometer or a photofluorometer is not available, a biological method is often preferred, the sensitivity and specificity of other chemical methods being essentially lower Finally it may be of value to use a biological method as a check on a physiochemical method, particularly with regard to specificity

It has therefore been considered of interest to describe a modification of the biological method of VANE (1957) by which it is possible to eliminate the spontaneous activity of the rat fundus strip thereby considerably increasing the accuracy of the biological method

Methods

As bathing solution modification of the rat uterus Ringer is used (GADDUM, PEART & VOGT 1949), (NaCl 7.2 g, KCl 0.42 g, $CaCl_2$ 0.06 g, $NaHCO_3$ 3.0 g, glucose 0.5 g to 1 litre with demineralized H_2O). This solution is aerated with carbogen (4% CO_2 + 96% O_2) for at least half an hour before setting up of the preparation, to give pH of 7 72. Throughout the experiments the solution in the reservoir as well as the organ bath is aerated. Scopolamine hydrobromide 0.1 mg base per litre, and mepyramine maleate 1 mg base per litre, is

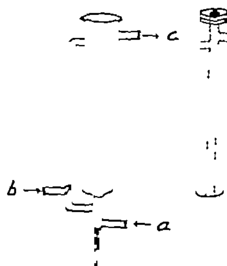


Fig. 1 Diagram showing the organ bath and the perspex tube which reduces the volume of the bath to 5.5 ml. a) Bathing solution. b) Carbogen. c) Suction at wash.

added in order to block the effect of acetylcholine and histamine respectively Scopolamine has, in addition, a restrictive influence on the spontaneous activity (cf. VANE 1957). The temperature of the organ bath is 37° The organ bath is an overflow vessel provided with side pipes to enable aeration (HANDOVSKY H personal communication to the director of this department). The aeration will cause circulation with downward current round the organ. The volume of the bath is reduced to 5.5 ml by means of perspex tube (fig. 1) (Ove SCHOU unpublished). To record the movements of the fundal-strip an isotonic pendulum lever is used (PATON 1957) with a 16 fold magnification and 1.0 g load increase per 2.0 cm deflection of the writing point. Standard solutions are made by dissolving 5-HT-creatinine sulphate (Sigma Chem. Co.) in a solution of the composition NaCl 9.0 g, ascorbic acid 10 mg, HCl 10.0 ml 0.1 N to 1.00 litre with demineralized H_2O

A fundal strip, about 7 cm long and 1–1.5 mm wide, is prepared as described by VANE (1957), stretching of the strip being avoided. Immediately after suspension in the organ bath, the strip is stretched *slowly* by steadily increasing load (worm drive) to a maximum of 1 0 g. The load in the position of rest (the baseline) is about 0.8 g and does not exceed 1.0 g. In contrast to Vane s method, no extra load is applied for stretching the preparation before each contraction. After each contraction, three washings are undertaken at intervals of 10 seconds. In the remaining period between two contractions, washings are performed every 30 seconds. At washing, the solution in the organ bath is exchanged by overflow. A contact time of 60 seconds is used with a cycle of 5 minutes.

Results

4-Point-assay

With the procedure described at least 9 out of 10 fundal strips show a rectilinear log-dose/effect-curve in the concentration range 0.2 1.5 ng 5-HT base per ml. The spontaneous activity is negligible and the resting

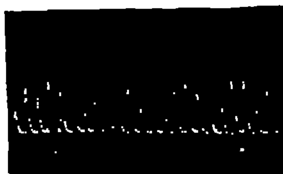


Fig. 2. Kymograph trace of a 4-point-assay showing that the spontaneous activity is negligible and that the resting length is almost constant.

length constant for 4–5 hours. The preparation is thus well suited for 4-point-assays (fig. 2) On one preparation a 4-point-assay (6 groups) was performed of two "test solutions" containing 30 and 50 ng 5-HT per ml, after which the results were subjected to analysis of variance and the contents calculated (table 1). The standard solution contained 40 ng 5-HT base per ml. *The solutions were added to the organ bath in 50 and 100 µl* portions.

Table 1

The results of 4-point-assays on known "test-solutions" and analysis of variance of the results.

Assay	ng 5-HT S_1	S_2	T	T'	Fiducial limits	Found content in per cent of true
1	2	4	1.5	3	92 % and 109 %	102.5
	2	4	2.5	5	95 and 106 %	104.4

Nature of variation	P 1	2
Between doses	<0.001	<0.001
Between groups	<0.001	<0.05
Regression	<0.001	<0.001
Parallelism	>0.1	>0.1

5 HT content in mouse skin

The following method was used for determination of 5-HT in mouse skin (cf. SECHER HANSEN LANGGAARD & JANSEN 1967). A piece of skin (corium + subcutaneous tissue) weighing about 250 mg was homogenized in 5 00 ml of the same solution as used for making the standard solutions. The homogenate was boiled for 20 minutes, cooled by standing and centrifuged at 1200 G for 30 minutes The supernatant was isolated by decanting and was applied directly to the fundal strip or after dilution. By adding the 5-HT antagonist BOL 148 (2 bromo lysergic acid diethylamide) to the bathing solution it was established that contractions caused by this test solution were due to 5-HT alone. In order to examine whether substances are found in mouse skin (for instance catecholamines), which would inhibit the effect of 5-HT in the test solution, a quantity of 5-HT corresponding to 50 % of the contents, was added to part of the solution. The recovery was 97 %, indicating that such an inhibition did not occur When 5-HT was added to a piece of tissue before homogenization, corresponding to the tissue s own content, 96 % was recovered, indicating that no loss occurred during the homogenization and boiling. In normal mice, a 5-HT content of 1 35 µg per g skin was found (SD = 0 70, n = 16).

Summary and Conclusion

A modification to Vane s method is described for the assay of the 5-HT in biological fluids and tissue extracts. By this method, 5-HT concentrations as low as 20 ng per ml are determined with an accuracy which is normally greater than 90 %. For the assay about 900 µl of a solution containing 20 ng 5-HT per ml are required.

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Evaluation of the Antitussive Effect of Noscapine and Codeine on Citric Acid Cough in Guinea-Pigs

By

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(Received December 13 1966)

The antitussive effect of noscapine (formerly designated as narcotine) has been studied in numerous clinical and animal experiments. Many investigations have shown that the evaluations of cough suppressants in man are unreliable (GRAVENSTEIN *et al* 1955 PRIME 1961) Man is better able to control his coughing voluntarily than animals so that critical evaluation of the real antitussive effect compared with the placebo is therefore unsatisfactory Furthermore, it has been difficult to develop a reliable and harmless technique for eliciting cough in animals. A number of methods have been tried using electrical, mechanical and chemical stimulation of the cough reflex in animals (ROSIERE *et al* 1956).

The results of the studies on the effectiveness of noscapine as an antitussive are variable. REICHLE & FRIEBEL (1955) have reported that the ED50 of codeine and noscapine tested as antitussive compounds in guinea pigs is 8.0 and 18.5 mg/kg, respectively yet according to WINTER *et al* (1954) codeine and noscapine have equal antitussive activity in guinea-pigs. Similar results in cats were reported by KONZETT (1955) Recent studies by LA BARRE (1959) show that noscapine is about 50 % more effective than codeine as an inhibitor of the centrally stimulated cough reflex in cats. HAHN & FRIEBEL (1966) have studied the effect of various antitussives on the cough reflex centres in the central nervous system

REICHLE & FRIEBEL have used a sulphur dioxide-air compound as a cough eliciting irritant. We tried sulphur dioxide for the induction of cough in guinea-pigs but it was not sufficiently irritant and increasing the sulphur dioxide concentration caused dyspnoe and profuse bronchial secretions. Another method was therefore investigated. BICKERMAN *et al*

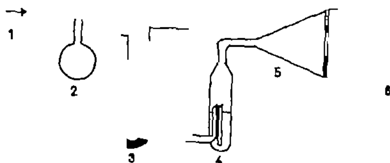


Fig. 1 The apparatus used in this work.
1 Air stream. 2. Gas flow meter 3 Hg manometer.
4 Nebulizer 5. Funnel. 6. Cage.

(1954) used nebulized citric acid as a bronchial irritant in their clinical experiments, and when we tested citric acid on the guinea-pigs it proved to be a very effective and harmless cough irritant.

In this work we have evaluated the antitussive effect of noscapine and codeine by using inhaled citric acid aerosol as an artificial cough eliciting agent in guinea-pigs.

Methods

Three and a half months old male guinea-pigs weighing 425–600 g (average 513 g) were used. At the end of the experiments they were 5 months old and weighed on average 610 g (445–760 g). The guinea-pigs were weighed each week before treatment.

The interval between experiments was one week, during which the animals were allowed to rest. The guinea-pigs were given 70 g of swede and 20 g oats per animal, and hay and water ad libitum.

Both codeine and noscapine in doses of 30 and 70 mg/kg were injected into the rectus abdominis muscle of the animals. 45 min. later they were exposed to the nebulized citric acid in the pparatus shown in fig. 1 Air was passed into the nebulizer at a constant pressure at the rate of 15 l/min. The citric acid aerosol which was produced then passed into the funnel adjoined to the cage and was inhaled by the animals. The back wall of the cage was adjusted according to the size f each test animal, so that the animal could only inhale the citric acid aerosol. The number of coughs were registered during an irritation period of 5 min.

Guinea-pigs which responded to the nebulized irritant (54) by coughing were selected. Two groups of 17 animals were used for testing the drugs and one group of 20 animals was kept as control group.

Crystalline citric acid dissolved in saline (175 mg/ml), and codeine phosphate and noscapine chloride each in 3 solution in saline were used. In the following are indicated the amount of noscapine and codeine as their corresponding salts.

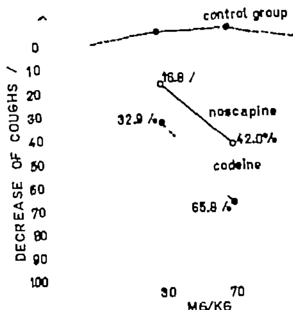


Fig. 2. Decrease of the number of coughs (%) after administration of noscapine and codeine in doses of 30 and 70 mg/kg.

Results

The antitussive effect of codeine in both doses was more marked than that of noscapine (fig. 2). Codeine 30 mg/kg decreased the total number of coughs by 32.9 % and noscapine 30 mg/kg by only 16.8 %. A dose of 70 mg/kg codeine decreased the number of coughs by 65.8 % and the corresponding dose of noscapine, by 42.0 %. With a dose of 30 mg noscapine, not a single animal completely stopped coughing. The total number of coughs varied in the range 1–17 during the 5 min. observation period. On the other hand, with same dose of codeine, 8/34 animals stopped coughing. The number of coughs varied between 0 and 20. The results with 70 mg/kg in the noscapine group were 3/34 stopped coughing and the number of coughs noted were 0–12/5 min. In the codeine group (70 mg/kg) the results were 11/34 and 0–9/5 min.

The basal coughing level in the group without drugs was the same at the beginning as at the end of the 6 weeks examination period. Before the experiments with the drugs, the number of coughs were 1–20/5 min. and after the experiment 1–16/5 min. The number of coughs in the control group varied during the six week period from 1 to 17/5 min.

Most of the guinea-pigs continued coughing after the citric acid irritation had finished and the animals had been taken out of the cage. These

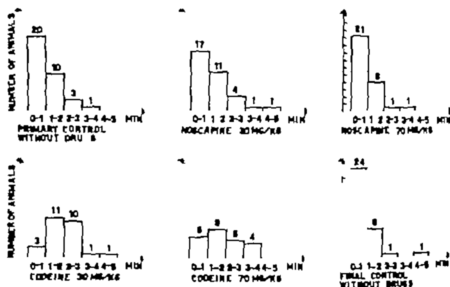


Fig. 3 Distribution f animals (34) according to the time at which they began coughing after citric acid irritation was started.

after-coughs were recorded during the following 5 min. The number of after-coughs was higher in the noscapine groups, than in the codeine groups.

The animals receiving codeine behaved more peacefully than those receiving noscapine. The guinea pigs gained weight normally throughout the investigation.

La Barre *et al.* (1959) reported that cats receiving noscapine began coughing later than the animals receiving codeine. In this work the time from the beginning of the citric acid irritation to the first cough was noted. Most of the untreated animals in the primary and final control groups started coughing during the first minute (fig. 3) The guinea-pigs in the noscapine group began coughing earlier than those in the codeine groups. There were no differences with regard to the starting point of coughing between the two doses of the drugs.

Discussion

Hahn & Friebel (1966) have reported that a definite decrease in the coughing of guinea pigs was seen only when relatively high doses of codeine (about 15–30 mg/kg) were used. In this work we used codeine and noscapine in doses of 30 and 70 mg/kg. The critical evaluation of the antitussive effect was difficult with smaller doses of the drugs. One reason

for the high doses was the use of citric acid which proved to be a very strong cough-eliciting irritant.

Noscapine showed a weaker antitussive activity than codeine. It is well known that the first phase in the physical dynamics of cough is a sharp deep inspiration. Some investigators have reported that noscapine has a mild spasmolytic effect on smooth muscle including the bronchi. Codeine, on the contrary is a bronchoconstrictor Noscapine therefore allows an effective, deep inspiration. And the expulsive phase of cough is then stronger Codeine acts as a depressant of the central nervous system. Noscapine in doses within the therapeutic range, probably has no effect on the central nervous system. Codeine is an antitussive which acts principally by direct depression of the cough center Noscapine is eliminated rapidly from the blood stream following intravenous injection, and it then becomes fixed in various tissues including the lung, liver and kidney of the cat. The concentration in the lung is almost twice that in the blood stream (COOPER *et al* 1934) In addition, noscapine has anticholinergic actions (TANAKA 1961) The site of action of noscapine may be in the afferent reflexogenic zones of cough centre and in the receptive elements of the lung.

In this work the animals receiving codeine almost completely ceased coughing after the citric acid irritation had stopped The animals in the noscapine group had more post-coughs. Codeine diminishes ciliary activity and has a "drying" action on the respiratory tract mucosa, which may explain the difference between the two drugs in the post-cough phenomenon.

Summary

Citric acid aerosol was found to be suitable in eliciting artificial cough in guinea-pigs. The technique proved to be satisfactory in the evaluation of cough suppressants. The antitussive activities of noscapine and codeine were compared in two doses of 30 and 70 mg/kg. Codeine was found to be more effective Noscapine unlike codeine did not delay the starting point of coughing.

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From the Home Office Forensic Science Laboratory Harrogate

The Production of Cyanide in Post Mortem Material

By

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Death from oral ingestion of cyanide is usually so rapid that significant amounts can be found in the stomach contents. Usually no further analyses are performed and hence despite the long history of cyanide poisoning, there are very few data (Bonnichsen & Maehly 1966 Curry 1963a Halström & Møller 1945 Sunshine & Finkle 1964) on the distribution of cyanide in tissues. In the course of many years experience of systematic forensic toxicological analysis we have come to the conclusion that, even in the presence of a reliable history as many tests as possible should be made for common poisons. It was while developing a scheme of routine toxicological analysis that we found by chance, a very faint smell of cyanide in a distillate from an intestinal content. (It has since been shown that one of us can smell cyanide in stomach and intestinal contents at concentrations of below 1 µg/ml.) This led us to investigate more suitable methods for cyanide analysis and that of Gettler & Goldbaum (1947), with minor modifications, was the one subsequently used in these investigations. The method involves blowing out any hydrogen cyanide in the sample on to a fixed small area of a filter paper impregnated with freshly prepared (in situ) ferrous hydroxide. After the completion of the formation of ferrocyanide, the paper is dipped in 50 / w/v hydrochloric acid, containing a trace of ferric chloride, which shows cyanide as a stain of Prussian Blue Comparison with stains between the range 1–5 micrograms allows of identification and estimation. Identification of cyanide is of great importance in this investigation and, in our opinion, this method is highly specific. It could however be argued that the addition of trichloracetic acid to the test medium before the passage of nitrogen used for blowing out hydrogen cyanide could bring about the decomposion of an intermediate cyanide complex but on several occasions the characteristic smell of cyanide was also detected, and we are convinced that it is hydrogen cyanide that has been produced in the biological systems described below The presence of cyanide was confirmed in the original distillate from the intestinal

contents, but the amount (about 0.5 milligrams) did not seem reasonable as the victim had apparently died from acute barbiturate poisoning. We began to use the method for examining blood samples, which at that time had been in the refrigerator and discovered one sample, number 544/59 which contained 3 µg/ml This was in the blood of a subject who had apparently died from barbiturate poisoning. As far as could be found in the literature the production of cyanide in post mortem blood could not be expected to reach such levels (GETTLER & BAINE 1938), but since we were not convinced we began a comprehensive series of experiments in an attempt to discover how cyanide had appeared in the intestinal contents and blood.

Experimental

One of the early theories considered was that bacteria could be responsible for the phenomenon. At a later stage Professor F Lundquist drew our attention to a paper by PETTY (1921), and it was then obvious that the significance of this very important paper had not been realised by forensic toxicologists. Petty suggested that Pseudomonas pyocyanea could produce cyanide and a further study of this particular observation was included in our series of experiments.

For the first stage of the experiments we used the blood (544/59) to incubate blood samples which did not contain cyanide and to follow the cyanide concentrations over a period of time. It was also obviously desirable to try and determine the rate at which cyanide production occurred, whether the phenomenon was aerobic or anaerobic and whether it could be prevented. We were hampered in our experiments as the original source of blood (544/59) was of small volume and was soon exhausted. However we were able to show that incubation of Pseudomonas with blood not containing cyanide did not enhance the production of cyanide and that fluoride inhibited its production.

A survey of these results is shown below

Table 1

Case Number	Dates	Cyanide Level µg/ml	
Blood Samples			
518/59	9th July 1959	nil	
518/59	23rd September 1959	5	
518/59	4th April 1960	0 1	
544/59	19th July 1959	2–4	
544/59	13th October 1959	40	
518/59 10 ml 544/59 0.5 ml	incubated at 4 from 9th July 23rd September	2.5	all in 4 refrigerator
518/59 10 ml 544/59 0 5 ml	incubated at 4 from 9th July 23rd September with added sodium fluoride	0.00	

At this stage of the investigation it was decided to inoculate blood samples (from out-of date transfusion bottles) with the following various strains of bacteria –

Strep. pneumoniae	Staphyl. aureus
Aerobacter aerogenes	Staphyl. albus
Proteus sp.	Monilia sp.
Bact. coli	Strep. pyogenes (Lancefield Gp A)
Strep. pneumoniae ordinary	(Strep faecalis, B mycodis,
Strep. viridans	Micrococcus tetrogenus)
N catarrhalis	Pseudomonas pyocyaneus

These experiments consisted f inoculating triple parallel sets f 10 ml of blood in test tubes plugged with cotton wool at 0° at room temperature (bout 20°) and at 37° fo varying lengths of time of periods up to 68 days. The results of cyanide analyses from selected tubes were also examined in relation to the blood group of the sample. The blood samples remaining after a aliquot had been taken for analysis were re-examined bacteriol ogically The incubation f blood with added amounts of ammonium oxalate (mount 0.5 1.0, 1.5 2 0 2.5, 3.0 mg/ml), urea (0.5 1.0, 1.5 2.0, 2.5 3.0 mg/ml), trypsin 0.1 mg/ml), cysteine and NH_4 formate (same mounts) did not reveal any significant increase due to the addition of these substrates.

Experiments with serum and red cells with saline, in a limited series of experiments involving 9 samples, i no case showed cyanide production over period of two weeks at room temperature although one f series of 12 samples of the original blood produced cyanide in a concentration f 0.25 µg/ml. When l ounce samples of transfusio blood, in test tubes closed with cotton wool plugs, were allowed to stand for two months t room temperature, very high levels of cyanide were obtained – i e. of the order of 100 µg/ml, confirming the result obtained on 544/59 blood which, as shown in T ble 1 had increased to 40 µg/ml after 3 months t 4

Results

As a result of over 450 cyanide determinations, covering many aspects of out investigations on the possible influence of bacteria it has been possible to show that

1) Cyanide production can occur spontaneously in bacteriologically sterile blood it is not a constant phenomenon and so far no way of in ducing it has been found Toxicologically significant amounts can be produced within a few days.

2) Production can also occur in the brain, liver kidney and uterus as well as stomach and intestinal contents.

3) Production is completely inhibited by sodium fluoride in a concentra tion of 1 / w/v

4) There are probably two phenomena involved – one occurring as a result of blood enzymes, the other involving Pseudomonas Pyocyaneus which appears to enhance production slightly No other bacteria among those tested showed any significant increase in the amounts of cyanide production

5) Production by sterile blood samples is slightly better at room temperature than at 37° or 4 pseudomonas pyocyaneus incubation produces slightly more cyanide at 4 than at 37° or 20°

6) Under the best conditions for production so far observed, levels of the order of 1 μg/ml can be expected after about 2–3 weeks rising to many tens of micrograms per ml after 2–3 months even after 10 months at room temperature levels as high as 1 μg/ml have been observed.

Cyanide Production in Actual Cases

A young woman was found dead in circumstances which indicated that death was due to shock following the introduction of a weak solution of soap and chlorxylenol type disinfectant into the uterus by means of a syringe. The characteristic smell of this disinfectant was observed when the jar containing the uterus was opened and chemical analyses revealed all its constituents. 14 days later when the uterus was being re-examined a very faint smell of cyanide was also detected. Analysis showed after 14 days heart blood nil and femoral blood 1 μg/ml while after 23 days heart blood 1.2 μg/ml, and femoral blood 1.2 μg/ml.

Washings of the uterus and stomach wall as well as of the kidney brain and liver all showed cyanide in amounts between 1 and 10 μg from 2 g samples, but it was clear that the distribution within each organ was irregular Drainings from the kidney showed the highest concentration to be 5 μg/ml, while in the brain it was found that only the decomposing matter at the surface of the brain contained cyanide. Subsequently it was possible to select those areas which contained detectable cyanide, by visual inspection. The white matter still in good condition in the body of the brain, contained no cyanide. This was a most useful finding in that our previous experience of cyanide poisonings had shown that in such cases, cyanide should have been present if cyanide had been the cause of death. At this time, samples were taken at random from organs of other cases which had been kept in the refrigerator In all cases in which signs of putrefaction were seen, cyanide was obtained in amounts of up to and over 10 μg/ml. It was our impression that kidney tissue was particularly suitable for this type of production. At the time we thought it probable that an aerobic bacterial infection was responsible as a high proportion of the tissue samples in the refrigerator contained cyanide and it was as a result of this that our studies on bacteria and cyanide were actively pursued On reflection, we still do not know whether this cyanide was produced by bacterial action or enzymatically

Since we have been aware of this phenomenon, the problems associated with interpreting cyanide levels have become much more difficult. On one

occasion (in conjunction with R. H. Fox) in the case of a death on December 15th the samples were taken into the laboratory on December 21st. The stomach contents at this time did not smell of cyanide. On December 29th, however the stomach contents were found to contain 200 µg of cyanide. The blood level was then 1 µg/g and the liver 2 µg/g. Unfortunately the brain had not been submitted and no definite interpretation of the results was possible. However nitrogen was bubbled through an aliquot of the stomach contents for 2 hours until a fraction gave negative results for cyanide. One week later a similar fraction gave a positive result with an estimated concentration of 1 5 µg/ml. This clearly indicated cyanide production.

The lowest level of blood cyanide to account for death is probably of the order of 1 µg/ml (CURRY 1963b), but in this context one case, in which recovery occurred after a blood level of 7.5 µg/ml, is clearly worth recording. A man in a chemical works collapsed from accidental inhalation of HCN vapour at 8 45 a.m. At 9 00 a.m. he was given an intravenous infusion of thiosulphate/nitrite antidote and made a dramatic recovery within 10 minutes Both fluorided and unfluorided blood, taken before therapy gave the same value of 7.5 µg/ml as HCN

Discussion and Summary

It was tempting to ascribe cyanide production to bacterial contamination until it was discovered that production occurred in bacteriologically sterile blood The theory of thiocyanate oxidase converting normal thiocyanate to cyanide is attractive but as the normal range of thiocyanate in the blood is said to be only 3 1–25.5 µg/ml it seems unlikely that there is sufficient thiocyanate to account for all the cyanide found.

Notwithstanding the absence of a demonstrable mechanism we feel our results worth recording in that attention is drawn to this phenomenon which is of great importance to forensic toxicologists. The use of the *white matter of the brain, protected from aerobic conditions* and the use of freshly fluorided blood are suggested as means of minimising the difficulties involved in this phenomenon

Acknowledgements

We should like to thank Dr J T B Bain for referring the non-fatal case of cyanide poisoning to us and Mr R. L. G Osborne and Mr R. H Fox for their co-operation in some of the experiments.

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From the Research Division, Pharmacia AB Uppsala, Sweden

An Observational Method Estimating Toxicity and Drug Actions in Mice applied to 68 Reference Drugs

By

Dag E. S. Campbell and Wolfgang Richter

(Received January 2, 1967)

The increasing number of newly synthesized organic compounds within the pharmaceutical industry and the desirability of finding useful applica tions for these compounds, has stimulated the development of rapid, reproducible, and sensitive small animal test procedures. As a rule, simple observational methods in conscious animals are used to obtain informa tion about interesting biological effects of new compounds, before more elaborate instrumental methods are employed.

In spite of the obviously widespread use of observational methods, few publications exist on the applicability scope and technical details of such procedures. Standardized observational screening procedures in mice have been described by IRWIN (1959 & 1962), HERMANSEN (1959 & 1960), SMITH (1961) and BASTIAN (1961)

We have further developed the method of HERMANSEN (1959 & 1960), which is based on some unpublished American industrial procedures for the estimation of toxicity and observation of various symptoms. The technique described here, which has previously been presented briefly (RICHTER & CAMPBELL 1962), has been used routinely for five years. Results obtained with 68 reference drugs, exemplifying the main groups of pharmacodynamic action, are presented. The procedure enables identification of compounds with actions on the autonomic and central nervous system.

Materials and Methods

Animals

Unfasted albino mice of either sex, weighing 18–30 g, from the NMRI strain, were used.

Apparatus

For the measurement of rectal- and paw skin temperature, miniature thermistor of the thermometer type was used (RICHTER 1964). The temperature sensitive spherical tip has diameter of 0.75 mm. A Wheatstone's bridge calibrated for measurements between 22–42°

and a galvanometer were used for measuring the change in the resistance of the thermistor. Estimation of pupil size was made either by the naked eye or with the aid of a microscope with a magnification of 20 times. Illumination with light behind a square of opal glass (40 × 40 cm) was found to be essential in order to minimize reflexes in the cornea. Experiments were performed at 18–23

Drug administration

Compounds were administered intraperitoneally either as solution in 0.9 % saline or as suspension in 0.9 % saline containing 0.5 % methylcellulose, in a concentration of 2, 1, 0.5, 0.25 etc. g/100 ml. The volume administered varied between 0.1 to 1.6 ml per 10 g bodyweight.

Procedure

On the day of testing, animals were transferred from the animal house to the laboratory in their housing containers each containing ten mice. The animals were marked by colouring and distributed in groups of three into cylindrical glass containers (20 cm height and 20 cm diameter) without access to food or water. The animals were weighed and rectal- and paw temperatures in °C were measured.

The test compound was first injected i.p. into three mice at the dose level of 200 mg/kg. Within 15–30 minutes after injection, each animal was observed within its container for the following signs: ptosis, piloerection, Straub tail phenomenon, and decreased or increased motor activity. Thereafter each animal was successively taken out of the container. While keeping the mouse immobilized with one hand, it was examined for the following signs: salivation, lachrymation, mydriasis and miosis. It was then placed on a smooth surface and observed for the following signs: abduced hindlegs, head drop and ataxia. Loss of the righting reflex was considered to be present, when the animal, placed on its back, remained there for at least 30 seconds. Using HAFFNER's technique (1929) a small artery clip was then applied to the tail one cm from the base. The test was considered to be positive, when the animal did not bite the clip within 30 seconds after application. Thirty minutes after drug injection rectal – and paw temperatures were measured again. An increase of the paw- and of the rectal temperature of 2.0° or more, and decrease of rectal temperature of 2.0° or more, indicated drug action. No attention was given to decreased paw temperature.

Each mouse was regarded either as a reactor or a nonreactor to each of the listed signs. A reactor is mouse showing the presence of a sign which is unambiguously observed by a trained worker and which is not present in control animal. A reactor was indicated in the chart by the digit 1 non-reactor by a horizontal bar. A very strong reactor was indicated by the digit 2. Any group of mice with two or three positive reactors was considered reactive group.

T king into account the number of deaths among the animals given 200 mg/kg, further groups were injected and observed. Doses were taken from the scale (mg/kg): 1600, 800, 400, 100, 50, 25 12, 6, 3 etc. At least one high dose which killed all three animals and one low dose which produced no or only one reactor to any sign was included. On the average, five to six groups of animals were required per compound. Animals surviving the observation period of 30 minutes were controlled 4, 48, 72 etc. hours later until no further deaths occurred.

Expression of results

Toxicity results were btained as 0, 1 2, or 3 deaths per group of three mice. With few exceptions, the sequence of the number of deaths in adjacent dose groups was one of the three types shown in table 1 representing three approximate dose-mortality relations. By

Table 1

Relation between the LD50 value and the number of deaths in groups of 3 mice injected with adjacent doses from the fixed scale.

Sequence of deaths in groups injected with adjacent doses		Figures in the table are approximate LD50 values in mg/kg													
		Highest dose in mg/kg at which no deaths occurred:													
Deaths/group	Percentage deaths/group	1600	800	400	200	100	50	25	12	6	3	1.5	0.8	0.4	0.2
3–3–3–0–0–0	100–100–100–0–0–0	2400	1200	600	300	150	75	38	19	9	4.5	2.3	1.1	0.6	0.3
3–3– –0–0–0	100–100–67–0–0–0	2910	1460	728	364	182	91	46	23	12	6	3	1.5	0.8	0.4
3–3–1–0–0–0	100–100–33–0–0–0		1780	880	440	220	110	55	28	14	7	3.5	1.8	0.9	0.5

interpolation, an approximate figure for the LD50 was obtained for any observation time.

The occurrence of any sign t each dose was expressed in relation to the acute toxicity The highest dose level at which no deaths occurred 24 hours after injection was the highest dose at which definite signs could be observed. This latter dose was therefore chosen as reference level and designated as dose level no 1 The successive lower doses in the fixed dose scale were numbered 2, 3 4 etc. The quantitative relationship between the number of the dose level and the LD50 for the most frequent sequence of deaths 3-3-3-0-0-0 is as follows

Number of dose level	1	2	3	4	5	6	7	8	9	10
Dose expressed as percentage of LD50	67	33	17	8	4	2	1	0.5	0.3	0.1

A sign occurring t dose level no. 1 was designated 1 1 and sign occurring at dose levels no. 1 and 2 was designated 1 2 etc. By tabulating all signs for each compound, a sign profile was obtained. An example of protocol is given in fig. 1

Results

Toxicity

The magnitude of the approximate LD50 after intraperitoneal administration varied from 0 6 mg/kg for neostigmine to 3520 mg/kg for trimethadione, the most frequent LD50 figure being 150 mg/kg. As seen from table 2, toxicity values at 30 minutes and 24 hours after i.p. drug administration were identical in 57 out of 68 compounds and the 11 remaining drugs showed an increased toxicity (see table 3) Only six drugs gave toxicity increases beyond 24 hours after injection, i.e. 9 / of the drugs tested. None of the drugs produced toxicity increase beyond 72 hours

Of the 68 drugs 61 were given as solutions, and 7 as suspensions. These latter showed a toxicity which was generally lower than that of the soluble drugs, and their toxicity increased with time (see table 3). The reproducibility of the toxicity results is illustrated by the 16 double estimations in table 4 columns 2 and 3 and by the ten times repeated determinations on pentobarbital in table 5

Sign patterns

The signs observed with the drugs occurred either singly or in combination, forming more or less typical patterns The following drug categories were clearly recognized

1) *Sympathomimetics* characteristically showed piloerection and mydriasis in parallel at 2–7 dose levels. Some sympathomimetics also gave increased salivation and increased paw temperature.

[illegible] Acute [illegible] with observation, [illegible] Date: [illegible]
Tested by U.S

[illegible] DOES NOT DEVIATE FROM THAT OF THE CONTROLS

Compound *Ephedrine HCl* Room temp °C 20

Presented as: [illegible] [illegible] solution [illegible]

[illegible]

Further [illegible]: Convulsion; Writhing movements; Repeated jumps; Tremor; High gait; [illegible]

Before / After / Before / After / Before / After / Before / After / Before / After

[illegible] temperature with subtraction of [illegible]

Rectal temperature with subtraction of [illegible]

Fig. 1 Example of filled in standard form showing acute toxicity and sign pattern of ephedrine in nine groups of three mice.

Table 2

Acute toxicities and sign patterns of 68 reference drugs in mice.

In the first and second columns the approximate LD50 values are shown. In the third column the highest non-lethal dose for each compound is given and designated as effective dose level no. 1. In the following columns, the doses at which the different signs were observed, are noted in the

		Approx. i.p. LD50, 30 min., mg/kg	Approx. i.p. LD50, 24 h, mg/kg	Effective dose level no. 1 mg/kg	Increased paw temperature, 2°C 31	Decreased rectal temperature, 2°C 32	Increased rectal temperature, 2°C 33
Sympathomimetics	Naphazoline	91	91	50	–	1:5	
	Isoproterenol	440	364	200	4 5		1:4
	Noradrenaline	23	23	12	2:4		
	l-adrenaline	4.5	4.5	3.0	1:1	2.2	
	Ephedrine	300	300	200			
"Sympatholytics"	Azapetine	150	150	100	1 3		
	Guanethidine	110	110	50	2:2	1 1	
	Phentolamine	182	182	100	1:9	1 4	
	Isoxsuprin	150	150	100	1 8		
	Tolazoline	75	75	50	1 7		
	Phenoxybenzamine	880	150	100	1:5	1 1	
"Parasympathomimetics	Pilocarpine	300	110	50	1:2	3:4	
	Neostigmine	0.6	0.6	0.3		1 1	
	Carbacholine	3.5	3.5	1.5			
Parasympatholytics	Benactyzine	150	150	100			
	Diphemanil	91	91	50			
	Hyoscine methonitrate	300	300	200			
	Benzoyl-hyoscine bromide	91	91	50			
	Hyoscine bromide	600	600	400	1 1	1:1	
	Atropine	300	300	200	1 1	1 1	
Strong CNS-stimulants	Amphetamine	150	150	100			1 1
	Pipradol	150	150	100			

form of two figures. The first figure designates the highest, and the second one the lowest dose at which the sign occurred. Digits 1 2, 3, 4 etc. give the dose in fractions of the effective dose level no. 1 namely 1/1 1/2, 1/4, 1/8 etc. of dose level no. 1 Thus, for a compound with an effective dose level no. 1 of 100 mg/kg, 1:5 means, that the sign in question was observed at 100, 50, 25 12, and 6 mg/kg. The notation 1 1 means that the sign occurs only at the effective dose level no. 1

Ptosis	Salivation	Lachrymation	Mydriasis	Piloerection	Increased locomotor activity	Straub tail phenomenon	Righting reflex (r.r.) abolished	Head drop (with r.r. present)	Pos. Haffner (with r.r. present)	Decr locomotion (with r.r. pres.)	Abducted hindlegs (with r.r. pres.)	Unsteady gait
7	14	15	16	17	3	13	6	10	12	4	9	5
–	1 3	–	1 7	1 7	–	–	–	–	1 1	1 7	–	–
	1 4	–	1:3	1 4	–	–	–	–	–	1 4	–	
		–	1:2	1 2	–	–	–	1:2	–		1 2	–
	2 2	2:2	1.2	1:2	–	–	–	–	–	1 3	–	–
	1:2	–	1:5	1 6	1 1	–		–	–		–	1 1
–		–	–	–	–	1:1	–	–	1 2	1:2	1 1	1 1
–	–	–	1:1	1 2	–	–		–	–	1:2	–	–
1:5		–	–	–	–	–	–	–	1 3	1:5	–	–
–		–	–	–	–	–		–	–	1 1	–	–
–	–	–	–	1 3	–	–	–	–	–	1 2	–	–
1 5	–	–	–	–	–	–	–	–	–	1 6	–	–
–	1 4	–	1 5	1 3	–	–	–	–	1 2	1:5	–	–
	1:1	1 1	–	–	–	–	–	–	–	1 2	–	–
	1 4	1 4		–	–		–	–	–	1 3	–	–
	–	–	1 5	–	2 3	–	–	–	–	–	1:1	1 1
			1 5	1:1			–	–	–	1:2	–	–
	–	–	1 13	–		–	–	–	–	1 2	–	–
			1:13	1:2		–	–	–	–	1 2	–	–
–		–	1:12	–	2 2		–	–	1 1	1:1	1 1	
–			1:10				–	1:1	–	1 1	1 1	1 1
	1 2	–	1 3	1 6	1 6	–	–	–	1 3	–	–	–
		–	–	–	1 4	–	–	–	1 1	–	–	1 4

		Approx. i.p. LD50, 30 min., mg/kg	Approx. i p. LD50, 24 h, mg/kg	Effective dose level no. 1 mg/kg	Increased paw temperature, 2°C	Decreased rectal temperature, 2°C	Increased rectal temperature, 2°C
					31	32	33
CNS-depressants	Meprobamate	1200	600	400	1 1	1 3	
	Hydroxidion	440	440	200		1:2	
	Noallymal	600	300	200	1 3	1.2	
	Thiomebumal	75	75	50	–		
	Pentymal	300	300	200	–	1 1	
	Pent barbital	150	150	100	–	1.2	
	Phenobarbital	728	182	100		1 1	
	Chlordiazepoxide	>1600	364	200	2 4	1 2	
Major tranquillizers	Reserpine	150	150	100			
	Acepromazine	150	150	100	1:11	1:7	
	Chlorpromazine	*220*	*220*	*100*	1 7	1 6	
	Promethazine	150	150	100	1 6	1 3	
Strong analgesics	M rphine	600	600	400	–	1:2	
	Pethidine	150	150	100	1 1	1 1	
Antidepressive gents	Imipramine	150	150	100	1 1	1:2	
	Amitriptyline	75	75	50	1:1	1 3	
	Iproniazid	>1600	1200	800	3 4	1 1	
Weak analgesics	Codeine	182	182	100		1:2	
	Phenylbutazone	300	300	200	2 2		
	Acetylsalicylic acid	1460	728	400			
	Amidopyrine	300	300	200		1 2	
Convulsi es	Strychnine	1 5	1 5	0.8			1 1
	Pentetrazol	75	75	50		1 1	
Weak CNS-stimulants	Cocaine	75	75	50			
	Caffeine	300	300	200	1 1		
Anticonvulsives	Phensuximide	1200	1200	800		1 3	
	Diphenylhyda toin	>3200	1200	800		1:2	
	Trimethadione	>3570	3520	1600		1 2	

Ptosis	Salivation	Lachrymation	Mydriasis	Piloerection	Increased locomotor activity	Straub tail phenomenon	Righting reflex (r.r.) abolished	Head drop (with r.r. present)	Pos. Haffner (with r.r. present)	Decr locomotion (with r.r. pres.)	Abducted hindlegs (with r.r. pres.)	Unsteady gait
7	14	15	16	17	3	13	6	10	12	4	9	5
1 1	–	–	1 1	–	–	–	1 1	2 2		–		–
1 4	–	–	–		–	–	1 4	–		–	–	5 5
1 2	–	–	1 1	–	–	–	1:1	2 2		2 2	–	2 3
	–	–	–	–	–	–	–	–	–	–	1:1	1 2
–		–	–	–	–	–	1 1	2 2	–	2 2	2 3	3 3
1 2	–	–	–	–	–	–	1:2	–	–	–	3 3	–
–		–	–	–	–	–	1 1	–	–	–	–	2 2
1 1	–	–	–	–	–	–	1:1	–		2 4	2 2	2 2
–	–	–	–	2 3	–	–	–	–	–	1:2	1 1	1 1
1 8	–	–	–	–	–	–	1 1	2:4	–	2 8	2 7	–
1 4		–	1 2	–	–	–	1 1	2 4	2 4	2 6	2:4	–
1 3		–	1 6	5 5		–	–	1 1	1:4	1 5	–	–
		–	1:5		2 6	1 5	–	–	1:5	1 1	–	–
		–	1 2		1 2	1 2	–	–	1 2	–	–	
		–	1 3		–	–	1 1	–	2 2	2 2	2 2	–
–		–	1 4		–	–	1 1	–	2 2	2 2	–	–
1 3		–	–	–	–			–	1 1	1:3	1 1	–
1 1		–	1 1	1 4			–	–	1 1	–	–	–
			1 1		:2	–	–	–	1 1	–	1 1	–
		–			–	–		–	1 1	–	–	
1 2		–		2 2		–			1:2	1 2	1 2	–
					–		–	–	–	1 1	–	
			1 1		–		–	–	1 2	1 1	1 1	–
		–	1 1	–	1 1	–	–	–	–	–	1 1	1 1
1 2			1 1		–	–	–	–		1:2	–	–
				–	–		1:1	–	–	2 2	2 2	2:2
				1:2	–		–	–	–	1:2	–	–
1 2			1 2	1:			1 1	–		2 3	2 2	2:2

		Approx. i.p. LD50, 30 min., mg/kg	Approx. i.p. LD50, 24 h, mg/kg	Effective dose level no. 1 mg/kg	Increased paw temperature, 2°C	Decreased rectal temperature, 2°C	Increased rectal temperature, 2°C
					31	32	33
Local anaesthetics	Dimethisoquine	38	38	25	–	1 1	
	Dibucaine	38	38	25		1:1	
	Pramoxine	300	300	200	–		
	Tetracaine	46	46	25	–		
	Lidocaine	150	150	100			
	Procaine	220	220	100	–		
Plain muscle depressants	Aminophylline	300	300	200	1 2	1 1	
	Diph.-dim.-aminobut.[1]	91	91	50		1 2	
	Papaverin	150	150	100		1 1	
Skeletal muscle relaxants	Tubocurarine	0.6	0.6	0.4			
	Gallamine	19	19	12	3:3	1:1	
Antihistaminics	Antazoline	110	110	50			
	Tripelenamine	91	91	50	–		
	Chlorpheniramine	150	150	100		1.2	
	Diphenhydramine	75	75	50	1 1	1 1	
Varia	Serotonin	1200	1200	800	–	1 7	
	Histamine	2400	2400	1600		3 3	
	Apomorphine	220	220	100	1 3	1:2	

[1] Diphenyl-dimethyl-aminobutane

2) "*Sympatholytics*" includes here compounds which depress adrenergic activity in various ways. Most compounds gave increased paw temperature at many dose levels, as a typical sign. The adrenolytic activity of major tranquilizers also led to increased paw temperature at many dose levels. Tranquilizers were, however, easily distinguished by additional signs.

3) *Parasympathomimetics* all showed increased salivation. Neostigmine and carbacholine also caused lachrymation. Pilocarpine did not produce lachrymation, but produced paradoxically mydriasis and piloerection.

Ptosis	Salivation	Lacrymation	Mydriasis	Piloerection	Increased locomotor activity	Straub tail phenomenon	Righting reflex (-r.r.) abolished	Head drop (with r.r present)	Pos. Haffner (with r.r. present)	Decr locomotion (with r.r pres.)	Abducted hindlegs (with r.r pres.)	Unsteady gait
7	14	15	16	17	3	13	6	10	12	4	9	5
	–	–	1 1	–	–	–	–	–	–	1 2	–	1:1
	–	1:1	–	–	–			1 1		1:1	1:1	1:1
	–	–	1 1	–	–	–	–	–	–	1:2	1 1	1 1
	–	–	–	–	–		–	–	–	–	–	–
	–	–	–	–	–		–	–	1 1	1:2	1 1	–
	–	–	–	–	–	–	–	–	–	–	–	–
1 2	1 1	–	–	–		–	–	–	1:2	1 1	1:1	–
		–	1 3	–	–	–	–	–		1:1	–	1 1
–		–	–	–	–	–	–	–	–	1:1	–	–
	–	–	–	–		–	–	–	–	–	–	–
		–	–		–	–	–	1 1	1 1	1 2	–	1:1
–		–		–			–	–	–	–	–	–
		–		–	1 1	–	–		1 1	–	–	–
	–				1:1		–	–	1 1	–	–	–
–		–	1 2	–	1 1	–	–	–	–	–	–	1 1
7 7		7 7	–	2 7			–	1:1	–	1:6	–	1:1
				3 3	–		–		–	1 3		
			1 1	1 2			–	–	–	1 2	–	–

4) *Parasympatholytics* produced typically mydriasis in 5–13 dose levels, without any other marked signs.

5) *CNS-stimulants* Increased locomotor activity at 4–6 dose levels was the most typical sign for the strong central stimulants pipradol and amphetamine. Weak CNS-stimulants produced no characteristic signs.

6) *CNS-depressants* as well as some related anticonvulsants showed a combination of signs, all reflecting loss of muscle tone and co-ordination. Typically these signs formed a hierarchic order related to the dose. Loss of righting reflex occurred at the highest dose levels, followed by one or several of the following four signs decreased locomotor activity head

Table 3

Toxicity increase of compounds requiring an observation time exceeding 24 hours for determination of a final LD50 value.

Compound	Solution or suspension	Approximate i.p. LD50 mg/kg at various times after inj.				
		30 min.	24 hours	48 hours	72 hours	96 hours
Trimethadione	Susp.	>3520	3520	1460	1460	1460
Diphenylhydantoin	»	>3200	1200	440	364	364
Acetylsalicylic acid		1460	728	440	440	440
Meprobamate	»	1200	600	600	600	600
Phenoxybenzamine	»	880	150	110	110	110
Iproniacid	Sol.	>9600	1200	1200	1200	1200
Chlordiazepoxide	»	>1600	364	220	182	182
Phenobarbital*)	»	728	182	182	182	182
Neallymal*)	»	600	300	300	300	300
Isoproterenol	»	440	364	300	300	300
Pilocarpine	»	300	110	110	110	110

*) Given as solutions of the sodium salts.

drop, abduced hindlegs, and unsteady gait. The same signs were also shown by the major tranquilizers and by the antidepressants. In addition, however the tranquilizers produced several other signs and tricyclic antidepressants produced mydriasis.

7) *Major tranquilizers.* The psychoactive phenothiazines chlorpromazine and acepromazine were characterized by a unique combination of signs at 4–11 dose levels: increased paw temperature, decreased rectal temperature, decreased motor activity, ptosis, abduced hindlegs, head drop and loss of righting reflex. Reserpine did not produce any clearcut symptoms within 30 minutes observation time.

8) *Analgesics.* The strong analgesics morphine and pethidine showed a positive Straub tail phenomenon, unique for this group, positive Haffner test, mydriasis, and increased motor activity.

All the other drug groups, comprising antidepressants, weak analgesics, convulsives, weak CNS-stimulants, anticonvulsives, local anesthetics, plain muscle depressants, skeletal muscle relaxants and antihistaminics produced a variety of observable signs without forming any recognizable pattern. Most of these signs occurred at a single or a few high doses only but occasionally signs occurred at up to seven dose levels.

Table 4

Acute toxicities of various drugs in mice determined with our toxicity observation procedure compared with LD50 figures from the literature.

1	2	3	4	5	6
Compound	I.p. LD50, mg/kg Toxicity-observation proc.)		I.p. LD50, mg/kg Literature data		
D-Tubocurarine	0.6	0.6	0.5	–	0.14
Strychnine nitrate	1.5	1.1	1.0	–	1.7
l-Adrenaline	4.5	7.5	4.0	–	3.5
Amitriptyline	75	–	76	–	73
Diphenhydramine	75	–	84	75	83
Pentetrazol	75	91	92	–	90
Cocaine	75	–	150	–	–
Diphemanil	91	55	47	–	–
Tripelennamine	91	–	70	65	45
Chlorpromazine	110	220	92	–	120
Imipramine	150	150	115	–	–
Pentobarbital	150	150	130	155	123
Pethidine	150	–	150	–	
Chlorpheniramine	150	–	77		–
Pipradol	150	–	94		–
Azapetine	150		210	–	–
Amphetamine	150	150	120	–	–
Benactyzine	150	150	100	–	
Codeine phosphate	182	–	130	–	
Phenobarbital	182	220	340		235
Diphenylhydantoin	182	364	190	–	200
Chlordiazepoxide	182	364	268	–	–
Atropine sulphate	220	300	250	–	–
Procaine	220	300	230		124
Isoproterenol	300	–	300	–	500
Phenylbutazone	300	–	336	–	–
Acetylsalicylic acid	440	364	495	–	
Morphine	600	–	500	–	–
Meprobamate	600		710	–	800
Iproniazid	1200		690		–
Serotonin	1200	–	868		–
Trimethadione	1460	–	1800	–	–

) On the average, five to six groups of 3 mice have been used per compound for estimating the approximate LD50. Doses from the following scale (mg/kg) have been used 1600, 800, 400, 200, 100 50, 25 etc. When several figures are given, they indicate results from different tests or references.

Table 5

LD50 values (mg/kg, i.p.) of pentobarbital sodium estimated in four groups of three mice each on 10 different days.

Time after injection		
30 minutes	4 hours	48 hours
150	150	150
150	150	150
150	110	110
110	91	91
150	150	150
150	110	110
110	110	110
150	110	110
110	91	91
150	150	150

Discussion

Considering the extensive use of mouse screening techniques in most pharmaceutical industries, the scarcity of published material is astonishing. IRWIN (1959 & 1962) has given some details on such a procedure exemplified by some reference drugs. SMITH (1961) mentions an alternative scheme. BASTIAN (1961) presents a special observational technique also including drug modification of metrazol convulsions and gives results on 59 reference drugs.

Our findings show that drugs acting on the autonomic and central nervous system can be detected. With trained personnel, the risk of loosing active compounds within these groups is small. Further the relative strength of actions in these drug groups can be assessed. Other drug groups, even some with marked pharmacodynamic action, escape detection due to the abscence of observable signs.

In spite of the small number of animals used per compound, toxicity data obtained with our procedure were found to be of the same order of magnitude as those reported in the literature (BARNES & ELTHERINGTON 1964 USDIN & AMAI 1963 SPECTOR 1956), see table 4. The present method uses about 15 mice per compound whereas 20 different published i.p. LD50 determinations, chosen randomly were based on about 70 animals per compound. The agreement found is explained by the fact

that in general, the slope of the i.p. dose mortality curve is steep and that our dose spacing is sufficiently narrow for a good precision. Further the role of large numbers of animals in acute toxicity tests is usually over-emphasized, compared to that of many other factors (e.g. SELEKKO et al. 1963 FERGUSON 1962), usually not controlled. From the literature (SPECTOR 1956) it appears that the time of observation for determinations of acute i.p toxicity in mice varies from one hour to more than a week, the most frequent value being about 24 hours. We found an observation time of 24 hours sufficient to determine the final toxicity in 86%, and 72 hours sufficient to give final figures in all 68 drugs investigated.

A scheme with fixed and geometrically spaced doses, enables comparisons of the effects of drugs in relation to their toxicity irrespective of absolute doses. IRWIN (1959) used the dose scale 3 10, 30, 100 300 mg/kg, but we found this dose spacing too wide, giving too rough estimates of toxicity and a too scanty yield of symptoms. We found the dose scale 3, 6, 12, 25 50 mg/kg, proposed by SMITH (1961) more adequate. Still smaller dose intervals would involve difficulties in clearly delineating the dose levels which show symptoms and would again require larger numbers of animals per dose.

In observational mouse methods, the number of signs or symptoms recorded is usually large (e. g. 44 IRWIN 1959), in an attempt to increase the discriminating power of the procedure. It seemed to us, that recording fewer signs, preferably reflecting pharmacodynamic entities and representing good end-points would give more relevant information. The 16 signs listed in table 2 were found most valuable whereas the following signs were considered of minor importance Vocalization, irritability reactivity grip-strength, pinna reflex, corneal reflex, exophthalmus, defecation and postural peculiarities.

The information inherent in each sign has a multidimensional character expressable in terms of duration, intensity frequency and dose range. One simple quantitative measure of each sign is here obtained by fixing the time of observation, classifying intensity per animal in the form of reactor or non-reactor classifying frequency at each dose level in the form of reacting or non-reacting groups and by stating the number of dose levels giving reacting groups. This way of quantitating each sign by stating the number of dose levels is facilitated by our more narrow dose spacing as compared with that of IRWIN (1959) Also, by designating the highest non-lethal dose level as dose level no 1 (as a rule corresponding to the LD5), the dose range for each sign is presented in a form which has close relationship to the therapeutic index. This enables a simple quantitative comparison of various signs and various drugs.

As the yield of signs generally decreased from 30 minutes after drug injection, examination of animals has regularly been performed 15-30 minutes after medication.

The route of administration is always a matter of discussion. Administration of six water soluble drugs by the i.v., i.p., p.o., and s.c. route was made to compare the yield of signs. Subcutaneous injection yielded most, i.p. and p.o. less and i.v. injection the least signs. The latter route is thus not attractive. Compounds with low water solubility or compounds, which are not absorbed orally have to be given parenterally. Routinely we have used i.p. administration of drugs, but the s.c. route is worth study.

In the following, typical members of drug categories, producing observable autonomic and central effects will be discussed. The observable effects which *sympathomimetics* are expected to produce, comprise piloerection, mydriasis, salivation, increased body temperature, exophthalmus, and vascular constriction. Our procedure shows that piloerection combined with mydriasis are the most constant characteristics, followed by salivation and increased body temperature. The particularly strong calorigenic action of isoprenaline (MOORE & UNDERWOOD 1960) is manifested here by increased rectal and paw temperature. The surprising decrease of rectal temperature produced by naphazoline may be explained by its known antihistamine properties. The peripheral vasoconstrictor action of sympathomimetics is difficult to detect by paw temperature measurements in mice because of their normally low paw temperature. A high paw temperature was found in guinea-pigs in which the vasoconstrictor action of ephedrine produced a pronounced decrease in paw temperature (RICHTER 1964). The "*sympatholytic*" group is represented by compounds showing alpha adrenergic blocking activity, adrenergic beta receptor stimulant and anti-adrenergic action. The adrenergic blocking action of phenoxybenzamine, phentolamine, tolazoline, azapetine, and of the neuroleptica acepromazine and chlorpromazine was reflected by their capacity to increase paw temperature at many dose levels. The increase in paw temperature in mice is thus a very sensitive detector of alpha adrenergic blocking action because of the low paw temperature in untreated animals, giving a temperature span of 17° between body core and extremities. The vasodilator effect of the beta receptor stimulants isoprenaline and isoxsuprine and that of the plain muscle depressant aminophylline was also demonstrable by increased paw temperature. Most of the alpha blocking agents also produce ptosis, but at fewer dose levels. FIELDEN & GREEN (1966) stress the usefulness of ptosis as an indicator of sympathetic blockade in mice. *Increased paw*

temperature seems to us an additional and more sensitive sign (RICHTER 1964). The known initial sympathomimetic effect of guanethidine and tolazoline probably explains the absence of ptosis and the occurrence of piloerection in our procedure. Among the *parasympathomimetics* carbacholine gives the expected salivation and lachrymation. The vasodilator action reported in the literature (BOVET & BOVET NITTI 1948) was not reflected by increased paw temperature No sweating was seen, as sweat glands in mice and rats are restricted to the foot pads (HAGEMANN 1960) The routine recognition of miosis was found difficult. Neostigmine also gives the typical signs, but surprisingly only at the highest tolerated dose. This may be due to the fact, that enzymatic activity in small animals is very high and neostigmine therefore produces asymptomatic enzyme inhibition until almost lethal doses are reached. Pilocarpine produces, in addition to the typical salivation, some signs of adrenergic stimulation such as piloerection and mydriasis, probably reflecting its known ganglionic stimulating properties. The *parasympatholytic* drugs are detected by their well known mydriatic effect (PULEWKA 1932) without the presence of any other signs The specificity of the mydriatic effect is outstanding. The strong *CNS-stimulants* amphetamine and pipradol markedly increased locomotor activity The weak CNS-stimulants caffeine and cocaine escaped detection, the moderate increase of motor activity produced by these compounds being masked by the exploratory hypermotility (see e.g. FORST 1939 KNOLL 1961) caused by the transfer to the new test environment. The motor activity increasing action of weak CNS-stimulants, therefore, is better detected by making observations after the disappearance of exploratory hypermotility Clinically *CNS-depressants* produce sedation hypnosis and general anesthesia. The sedative and hypnotic action has not been detected in mice. Subanaesthetic and anaesthetic doses in mice result in unsteady gait, abduced hindlegs, decreased rectal temperature or loss of righting reflex. Of these signs, loss of righting reflex and unsteady gait are most typical. However they only appear at a few high dose levels *Chlorpromazine like* compounds produce a characteristic sign pattern in mice ptosis, decrease of body temperature and symptoms of muscle weakness such as abduced hindlegs, decreased motor activity and head drop In addition, the known adrenolytic properties of such drugs produce increased paw temperature, due to peripheral vasodilation at low doses. This combination of well defined and independent signs allows detection of chlorpromazinelike compounds without the use of more elaborate behavioural characteristics, as used by IRWIN (1959) The activity of *strong analgesics* is revealed by the Straub tail phenomenon, which is the most specific symptom for this group and

also by a positive Haffners test. The known strong excitatory action of morphine like analgesics in mice (FORST 1939) resulted in increased motor activity. The correlation between the mydriatic and analgesic effects of morphinelike drugs in mice is well known (JANSSEN & JAGENAU 1956) and was also found with the present procedure.

Summary

A simple observational procedure in mice for the estimation of toxicity and detection of drug effects in new compounds is described.

16 different symptoms or signs were chosen as indicators of various pharmacodynamic actions. A simple way of quantitation of each sign is used, enabling the information about the different actions of each compound to be presented in a concise manner. This also facilitates comparison of different signs and drugs.

The procedure was applied to 68 reference drugs. The following categories showed typical sign patterns: Sympathomimetics, sympatholytics, parasympathomimetics, parasympatholytics, strong CNS-stimulants, CNS- depressants, chlorpromazine like compounds and strong analgesics. Other drug categories, even with marked pharmacodynamic actions did not produce characteristic, observable signs.

Use of this sensitive observational method for the screening of new compounds will give an approximate LD50 figure and will detect and quantitate autonomic and central drug effects.

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Studies on the Destruction of Adrenaline in Nutrient Solution

By

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(Received September 9 1966)

Adrenaline (A) is rapidly oxidized spontaneously at an alkaline pH in the presence of oxygen. The amount of oxygen that is generally dissolved physically in a nutrient solution is sufficient for this oxidation, at least with a moderate catecholamine (CA) concentration. The oxidative destruction is accelerated by certain ions, e g. Cu^{++}

In studies on the effects of A on isolated tissue specimens, the specimen is often placed in a nutrient solution which contains, among others, a number of metal ions. This solution is then gassed (in aerobic experiments) with pure oxygen or an oxygen mixture. The potentialities for oxidative destruction of A thus appear to be very good. It may therefore be asked how long does an active A concentration remain in the tissue bath. This does not appear to have been studied with the more recent chemical methods of A determination.

Material and Method

Adrenaline was added to a tissue bath containing 15 ml Krebs-Henseleit bicarbonate buffer made up with glass-double distilled water and gassed with carbogen (95% O_2 and 5% CO_2) so as to give an initial concentration of 10^{-4} or 10^{-7} g/ml. The temperature of the bath was 37 and its pH 7 40. Half minute, 7 5 15 30 and 60 min. after the addition of A, 1 ml of the buffer was taken for nalysis of the A concentration. The sample was transferred immediately to mixture of 0.5 ml 0.4 N perchloric acid, 2 mg ascorbic acid and 10 mg EDTA (tetracemin NFN) and then diluted with double-distilled water to 5 ml The quantity of A was then determined spectrophotometrically according to HÄGGENDAL (1963) after any o idation products had been removed by elution through a column ith strong cation exchanger (Dowex 50 W X 8). No correction was made for loss during the quantitative determination (the recovery was est mated as about 80%). The determinations ere made both after the addition f A t nutrient solution alone, and after its addition to a solution containing a tissue specimen. This specimen consisted of rat diaphragm, weighing about 0.5 g/solution. The rats were either untreated or had been given 100 mg thyroxine per

animal for 7 days before being killed. After preparation, the rat diaphragm was transferred to flask containing buffer where it was incubated for 30 min. Immediately before A was to be added the tissue was transferred to another flask containing now buffer at 37° to which the A was added. From this flask the samples were then taken (table 1). In some experiments the tissue was added 15 and 30 min. before the addition of A (table 2). These values were corrected for recovery (85 %, table 2).

Results and Discussion

The results are shown in fig. 1 and table 1 and 2. In buffer alone very rapid destruction of the added adrenaline occurred in 3 cases out of 4. In one case the destruction was less marked (see table 1).

When the tissue specimen was present, the rate of desctruction was reduced. From the 15th minute onwards the A concentration was, on the whole, constant, and the concentration during this period was maintained at over 50 / of the original value both when this was 10^{-6} and 10^{-7} g/ml. The tissue from the thyroxine-treated rats did not differ from that of the untreated rats.

Some factor in the tissue thus appears to protect A from destruction.

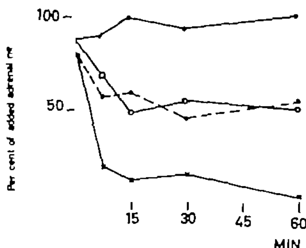


Fig. 1. Destruction of adrenaline (A) in nutrient buffer solution. Mean of A concentration in per cent of the initial A concentration 10^{-6} or 10^{-7} g/ml at different time intervals.
——×——× Buffer with no diaphragm added.
○——○——○ A and rat diaphragm added simultaneously to buffer solution.
●– – –●– – –● A and rat diaphragm from thyroxine-treated rats added simultaneously to buffer solution.
●——●——● A added to buffer which had been incubated with rat diaphragm 15–30 min. before the A addition.

Table I

Changes in adrenaline (A) concentration in bicarbonate buffer alone and in buffer with diaphragm from untreated and thyroxine-treated rats *added at the same time as A*. Initial A concentrations 10^{-6} and 10^{-7} g/ml buffer. Each column represents one experiment.

Time after A	(min.)	Concentration of A in µg/ml buffer			
		Initial concentration of A			
		1.00		0.100	
Buffer alone	0.5	0.87	0.64	0.102	0.056
	7.5	–	0.01	0.054	0.000
	15.0	–	0.01	0.042	0.003
	30.0	0.27	0.00	0.039	0.007
	60.0	0.09	0.00	0.007	0.003
Buffer + diaphragm from untreated rats	0.5	0.84	0.80	0.083	0.116
	7.5	0.77	0.90	0.048	0.039
	15.0	0.52	0.53	0.034	0.052
	30.0	0.63	0.66	0.029	0.039
	60.0	0.49	0.50	0.030	0.031
Buffer + diaphragm from thyroxinetreated rats	0.5		–	0.092	0.048
	7.5	–		0.062	0.051
	15.0	–	–	0.052	0.063
	30.0	–	–	0.045	0.044
	60.0	–		0.054	0.056

As early as 1922 MAIWEG found that the stability of A was increased after the addition of blood, which he ascribed to some protein with a protective action. It is possible that this factor may consist of some reducing substance or that it may bind metal ions of importance for the oxidation.

The occurrence of this phenomenon means that when a tissue specimen is present, the nutrient solution changes to such an extent as to influence the oxidative process. The initial rapid destruction of A may be assumed to be due to the fact that this factor has not yet been sufficiently given off to the buffer solution. This was further studied by adding A (in amounts as above) 15 and 30 min after the buffer solution had been incubated with the rat diaphragm. As shown in fig. 1 and table 2 no marked destruction occurred up to 60 min after the addition of A. In a control experiment with no diaphragm, the destruction of A was very rapid as in the first part of the present study

Table 2

Adrenaline (A) concentration in bicarbonate-buffer and in buffer solution which had been incubated for 15 and 30 min. resp. with rat diaphragm before addition of A. The values are corrected for recovery (85 %). For further information see table 1

Time after A	(min.)	Concentration of A in µg/ml buffer. Initial conc. of A: 1.00	0.100
Buffer alone	0.5	–	0.096
	7.5	–	0.021
	15.0	–	0.000
	30.0	–	0.005
	60.0		0.003
Buffer + diaphragm preincubated 15 min.	0.5	0.65	0.108
	7.5	0.91	0.083
	15.0	0.83	0.124
	30.0	0.76	0.100
	60.0	0.84	0.141 *)
Buffer + diaphragm preincubated 30 min.	0.5	0.96	0.082
	7.5	1.00	0.088
	15.0	0.92	0.099
	30.0	1.06	0.093
	60.0	0.89	0.097

*) This value appears to be too high of technical reasons.

It is possible that the capacity for protecting A from destruction varies with different tissue specimens.

Summary

The destruction of adrenaline (A) in initial concentrations of 10^{-6} and 10^{-7} g/ml in Krebs-Henseleit bicarbonate buffer gassed with carbogen (95 % O_2 + 5 % CO_2) was studied in the presence and absence of rat diaphragm. The A concentration was determined by a fluorescence method according to the principles of the trihydroxyindole reaction after purification with strong cation exchanger. An inhibitory effect on the destruction of A was demonstrated when rat diaphragm was present.

Diaphragm from thyroxine-treated rats did not differ in this respect from that of untreated animals. When rat diaphragm was pre-incubated 15 min before addition of A no destruction of A was observed.

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Tar Content and Ciliotoxicity of Cigarette Smoke

By

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In addition to their possible effects on the clearance of the respiratory tract and the lung, the ciliotoxic property of various compounds can be considered as an index of their cytotoxicity in general. It is thus natural that in the evaluation of the biological properties of tobacco smoke the ciliotoxic effect is of particular interest. This property of tobacco smoke has been studied using a number of different experimental designs and although it is generally agreed that tobacco smoke is ciliotoxic, there is controversy as to which component in the smoke is the most harmful. Certain reports indicate that the gaseous phase is the most ciliotoxic (KENSLER & BATTISTA 1963 GUILLERM *et al* 1961), others that the particulate phase which mainly consists of tar nicotine and phenol is the most ciliotoxic (BERNFELD *et al* 1964 FALK *et al* 1959)

In order to elucidate the significance of the tar content in cigarette smoke as a determinant of the ciliotoxic effect under exposure conditions which resemble human exposure, short term exposure experiments were performed using animal preparations *in vivo*

Methods

Full grown cats were anaesthetized and their tracheal cilia bserved by means of tracheotomy according to previously described technique (Dalhamn 1959). The bjective of microscope supplied ith an incident light was connected to the trachea by means of a rubber cuff. The motion of the cilia at the bottom of the trachea caused a flickering light reflex which was easily observed. A glass tube continuously heated to 37° and with volume equal to that of cat mouth was connected to the trachea just below the larynx and to respiratory pump supplying moist air The frequency of the pump was adjusted to the re spiratory rate of the animal.

Cigarettes with cellulose acetate filters giving varying amounts of tar in the smoke but an identical composition of the gaseous phase were used. The composition of the smoke was

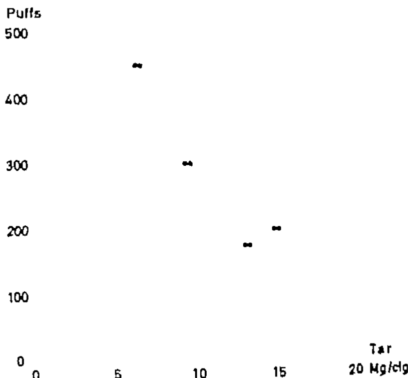


Fig. 1 Number of puffs required to arrest ciliary activity in trachea at various tar levels in cigarette smoke.

determined using methods described previously (Research Div P Lorillard INC, Spears 1963 Spears & Routh 1964). In addition the tar level expressed as TPM dry was determined (Schultz & Spears 1966) The cigarettes were connected to a syringe and 17.5 ml were drawn rather rapidly and 1 ml was immediately afterwards injected into the "mouth" of the animal. This "puff" was administered at every third breath and after 25 puffs the animal was allowed to rest for a period of 5 minutes. The number of puffs administered per unit time varied slightly according to the respiratory rate of the animal.

Results

Cigarettes of five different tar levels were used. Each type was administered to five different animals in a randomized manner in order to decrease the experimental error The number of puffs required to produce a complete arrest of the flickering light reflex, i.e. ciliostasis, in the whole area observed was noted. As no increase in the amount of mucus was present when ciliostasis occurred the possibility that the cilia could continue to beat under a stationary layer of mucus was excluded. Control animals exposed to air only had unaffected ciliary activity during a period equal to that required to produce ciliostasis in the smoke exposed animals. The results are shown in fig. 1 where for each tar level and each

animal, the number of puffs required to produce ciliostasis is given. An increase in the tar content of the cigarette smoke resulted in an increased ciliotoxicity i.e. fewer puffs were required to arrest the cilia.

Discussion

The smoke exposure technique involves the administration of standard ized amounts of cigarette smoke, produced under identical conditions from the different cigarettes. The smoke dosage apparatus previously described (DALHAMN *et al* 1963) is not suitable for the present type of experiment as the piston device in the apparatus causes a small leakage of air into the smoke. As variations in the tar level between the different cigarettes used in this experiment are associated with variations in pressure drop across the cigarette when sucked, the leakage and hence the concentration of smoke would be different for the various tar levels if this apparatus was used. Previous comparisons between the syringe administration technique as used here and the smoke dosage apparatus showed that no difference could be detected in the effects registered. (DALHAMN & RYLANDER 1966).

Although the results of the present experiments indicate that tar is an important determinant for the ciliotoxic properties of smoke, it appears possible that the gaseous phase could be more important if other exposure methods were used. This applies particularly to experimental models where the normal mucus layer of the trachea is not present, e.g. when *in vitro* preparations are used. As to conclusions concerning human exposure conditions, however an exposure model which resembles as near as possible the exposure conditions present under normal smoking appears to be desirable.

Moreover preliminary results of experiments on the absorption of various components of cigarette smoke in the mouth of human smokers (SPEARS 1965) indicate that the gaseous components are to a large extent absorbed within a very short time. Although it has previously been re ported (DALHAMN & RYLANDER 1966) that the amount of gaseous matter in the smoke is also a determinant of the ciliotoxic properties, the results of the last mentioned experiments suggest that the particulate phase might be more important for the biological effects encountered during smoking in man.

The results of the experiment reported here also stress the importance of determining the exact composition of the tobacco smoke when exposure experiments are carried out. Rerefences to "tobacco smoke" or "filter" and "non-filter" cigarettes do not give the necessary information about the exposure agent.

Summary

The ciliotoxic property of cigarette smoke with varying tar content was examined in short-term exposure experiments using animal *in vivo* preparations. An increased toxicity was noted when the tar content increased. The possible significance of this finding in connection with human exposure to tobacco smoke is discussed.

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Interference of Phenoxybenzamine and Guanethidine with the Vasoconstrictor Responses of Noradrenaline and Angiotensin II in the Hand

By

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(Received April 24, 1967)

The mechanism of action of angiotensin is complex. At one time the direct smooth muscle stimulating effect of the drug was considered to be the main determinant of its pharmacological effects. However during the last few years a number of other actions of the drug have been revealed (review by DOUGLAS 1965). Animal studies recently described indicate that the vasoconstrictor and hypertensive effect of angiotensin is not entirely due to a direct action on vascular smooth muscle but is also partly dependent on an indirect effect on the cardiovascular system mediated via the sympathetic nervous system (BICKERTON & BUCKLEY 1961 HALLIDAY & BUCKLEY 1962 LAVERTY 1962 & 1963 ZIMMERMAN 1962 MCCUBBIN & PAGE 1963 MCGIFF & FASY 1964 BENELLI DELLA BELLA & GANDINI 1964 VOGIN & BUCKLEY 1964 YOUMANS *et al* 1964 SMOOKLER & BUCKLEY 1965)

From studies on animals there is evidence for both a peripheral (ZIMMERMAN 1962 MCCUBBIN & PAGE 1963 YOUMANS *et al.* 1964 BENELLI, DELLA BELLA & GANDINI 1964) and a central site of action in the sympathetic nervous system (BICKERTON & BUCKLEY 1961 HALLIDAY & BUCKLEY 1962 LAVERTY 1962 & 1963 VOGIN & BUCKLEY 1964 SMOOKLER & BUCKLEY 1965).

In the present study an attempt was made to analyze the mechanism of the vasoconstrictor action of angiotensin in the human hand. This was done by comparing the effect of the peripherally acting sympathetic blocking agents, phenoxybenzamine and guanethidine, on the vasoconstrictor effects of intravenously or intra-arterially administered noradrenaline and angiotensin. Some of the results have been reported previously in a preliminary form (JOHNSSON, HENNING & ÅBLAD 1965).

Methods

The experiments were performed on healthy students at a room temperature of 27 ± 0.5° A thin polyethylene or teflon catheter was introduced into one brachial artery as previously described (ÅBLAD, JOHNSSON & HENNING 1961) and through it phenoxybenzamine, guanethidine, noradrenaline, angiotensin or isotonic saline was infused at a constant low rate (0 6 ml/minute) using a motor-driven syringe. The intra-arterially administered drugs were given in low doses which only affected the blood flow of the ipsilateral hand (test hand), while the other hand served as a control (control hand). Infusions of noradrenaline and angiotensin were also made into a superficial arm vein. The blood flow of the hands was recorded by venous occlusion plethysmography once or twice a minute. Details of the plethysmographic method have been described previously (ÅBLAD, JOHNSSON & HENNING 1961 & 1962).

In another series of experiments the mean arterial blood pressure after noradrenaline and angiotensin was recorded on a Grass Model 5 Polygraph through a teflon catheter in *the brachial artery connected to a Statham strain gauge transducer*

Most of the experiments proceeded according to the following pattern. When the blood flow and blood pressure had reached a stable level, noradrenaline or angiotensin was infused intravenously or intra-arterially for five minutes. The interval between the administration of the drugs was always sufficiently long for the effect of one drug to disappear before the next one was given. Following the vasoconstrictor tests, phenoxybenzamine or guanethidine was infused intra-arterially for five minutes (test hand). When the vasodilating effect of these drugs had reached a constant level (cf. HENNING & JOHNSSON 1967) the test procedure with the vasoconstrictor agents was repeated. In a few experiments only one of the vasoconstrictor drug was given. The results of the experiments were similar whether two or only one vasoconstrictor drug was used. Moreover the results were qualitatively the same, independently of the doses used.

The drugs used were l-noradrenaline bitartrate given intravenously in a mean dose of 18 µg/minute (range 14–20 µg/minute and in one experiment 10 µg/minute). When administered intra-arterially the mean dose of noradrenaline bitartrate was 0.31 µg/minute (range 0.22–0.44 µg/minute). The mean dose of intravenously infused angiotensin (calculated as the amide, hypertensin ®, Ciba) was 2.2 µg/minute (range 1 9–2.4 µg/minute and in one experiment 4.2 µg/minute). The corresponding intra-arterially administered dose of angiotensin was 0.48 µg/minute except in one experiment, in which the dose was 0.36 µg/minute. Phenoxybenzamine hydrochloride (dibenzyline ® Smith, Kline and French) was given in a total mean dose of 0.09 mg (range 0.02–0.20 mg) and guanethidine bisulphate (ismelin ® Ciba) in a total mean dose of 1.5 mg (range 0.5–2.0 mg). The doses of intravenously administered noradrenaline and angiotensin respectively were of the same order of magnitude in each of the three series A, B and C as shown in the results.

The recorded basal values are means of determinations made over two-minute periods immediately before the application of a vasoconstrictor stimulus. The values representing blood flow and mean arterial blood pressure changes in response to noradrenaline and angiotensin are means of determinations made during the last two minutes of the infusion.

Results are expressed as means ± standard errors of the means. The statistical analysis was based on the t-test (FISHER 1958) for differences in subjects. The analyses were performed on the differences between the absolute blood flow changes in the two hands induced by the different stimuli

Results

A. *Effects of intravenously administered noradrenaline and angiotensin on mean arterial blood pressure*

The effect of intravenously infused noradrenaline on arterial blood pressure was studied in 5 subjects (14 infusions of the drug were studied) and that of angiotensin also in 5 subjects (17 infusions) The basal mean arterial blood pressure was roughly the same in the two series, i.e. 90 ± 2.0 mm Hg before noradrenaline and 88 ± 1 9 mm Hg before angiotensin.

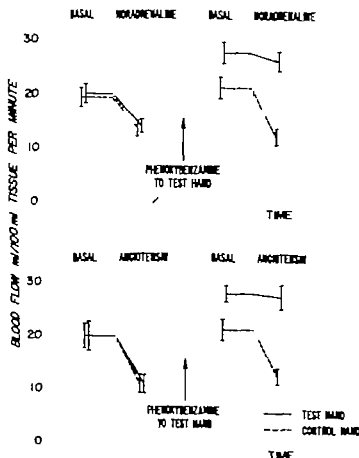


Fig. 1 Effect of intravenous infusion of noradrenaline (mean of 7 experiments) and of angiotensin (mean of 7 experiments) on blood flow in the hand before and after administration of phenoxybenzamine into the brachial artery on the same side as the test hand (solid line). The other hand served as control (dashed line). Mean ± s.e.m.

The corresponding values during intravenous infusion of noradrenaline and angiotensin were 114 ± 2.5 and 111 ± 2.5 mm Hg, respectively. Intra-arterial infusion of either of the drugs did not change the arterial blood pressure.

B *Effects of phenoxybenzamine on the action of intravenously administered noradrenaline and angiotensin in the hand*

The effects of intravenously administered noradrenaline and angiotensin on the blood flow in the hands before and after intra-arterial administration of phenoxybenzamine into the test hand were both studied in 7 experiments (fig. 1). After phenoxybenzamine, the blood flow gradually increased in the test hand and reached a stable level after about 30-60 minutes. The basal level of the blood flow in the hand and the increase after phenoxybenzamine were about the same in the studies with noradrenaline and angiotensin.

Before the infusion of phenoxybenzamine, *noradrenaline* produced a quantitatively similar reduction in blood flow in the two hands, the decrease in the test hand being 5.7 ± 1.30 ml/100 ml tissue/minute (in the following abbreviated as ml) and in the control hand 5.7 ± 1.71 ml (difference 0.0 ± 0.67 ml). After phenoxybenzamine had been given, the effect of noradrenaline on the test hand blood flow was much smaller than on the control hand blood flow, namely 1.2 ± 1.56 ml and 9.0 ± 1.74 ml respectively (difference 7.8 ± 1.16 ml, $p < 0.001$).

Before the administration of phenoxybenzamine *angiotensin* produced about the same decrease of blood flow in both hands. The decrease was 9.1 ± 2.00 ml in the test hand and 9.1 ± 1.24 ml in the control hand (difference 0.0 ± 1.11 ml). After the infusion of phenoxybenzamine, the effect of angiotensin on blood flow was much less in the test than in the control hand. The decrease was now 0.5 ± 1.93 ml and 8.5 ± 1.73 ml respectively (difference 8.0 ± 1.68 ml, $p < 0.01$).

C *Effects of guanethidine on the action of intravenously administered noradrenaline and angiotensin in the hand*

The effects of intravenously administered noradrenaline and angiotensin on the blood flow in the hands before and after intra-arterial administration of guanethidine were both studied in 7 experiments (fig. 2). In three experiments, guanethidine induced an initial decrease in blood flow followed by an increase in the test hand, while only an increase was recorded in the other experiments. The increase in blood flow had become stabilized in about 30 minutes. The basal levels of the blood flow in the hands and the

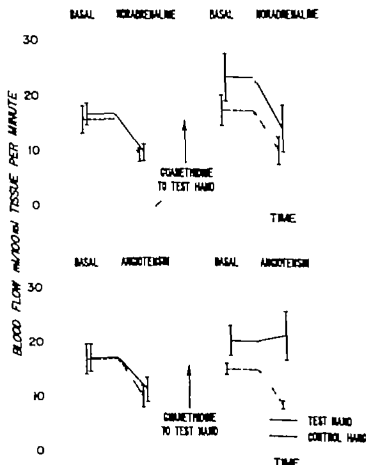


Fig. 2. Effect of intravenous infusion of noradrenaline (mean of 7 experiments) and of angiotensin (mean of 7 experiments) on blood flow in the hand before and after administration of guanethidine into the brachial artery on the same side as the test hand (solid line). The other hand served as control (dashed line). Mean ± s.e.m.

increase of the test hand blood flow after guanethidine were of the same order of magnitude in the studies using either noradrenaline or angiotensin and were comparable to these recorded in the study with phenoxybenzamine

The effect of *noradrenaline* on hand blood flow was essentially the same in the test and the control hand, before the administration of guanethidine. Thus there was a decrease of 7.0 ± 1.90 ml and 6.6 ± 2.22 ml respectively (difference 0.4 ± 0.63 ml) When guanethidine had been infused into the test hand, the decrease in blood flow after noradrenaline tended to be

somewhat greater in this hand than in the control hand, 9.3 ± 1.57 ml as compared to 7.2 ± 1 75 ml (difference 2.1 ± 0.57 ml $p < 0.02$).

Angiotensin caused a mean decrease of hand blood flow of 5.9 ± 1.81 ml in the test and 6 8 ± 2.10 ml in the control hand before the infusion of guanethidine (difference 0.9 ± 0 62 ml) After guanethidine, angiotensin caused a slight increase in blood flow of the test hand (1 0 ± 2.06 ml) The decrease in the control hand was roughly the same as that seen before guanethidine administration, 6.5 ± 1 54 ml (difference 7.5 ± 2.74 ml $p < 0.05$)

D *Effects of intravenously infused noradrenaline and angiotensin on the peripheral vascular resistance after phenoxybenzamine and guanethidine*

The following calculations were performed in order to obtain a rough estimate of the effects of intravenously infused noradrenaline and angiotensin on the vascular resistance in the hands after infusion of phenoxybenzamine or guanethidine into the test hand. The effects of the drugs on the mean arterial blood pressure (reported in A) and on hand blood flow (B and C) were calculated on a percentage basis These values and the percentage changes in peripheral resistance units (P R U corresponding to PRU 100 of GREEN 1948 calculated by dividing the mean of the mean arterial blood pressure by the mean of the blood flow in the hands) are

Table 1

Percentual changes of mean arterial blood pressure, hand blood flow and P.R.U. (P.R.U. = peripheral resistance units, correspondi g to PRU_{100} of Green 1948).
Mean ± s.e.m.

Drug		Increase mean arterial blood pressure per cent	Blood flow decrease per cent		P.R.U. Increase per cent	
Intravenously	Intra-arterially into test hand		Test hand	Control hand	Test hand	Control hand
Noradrenaline	Phenoxybenzamine	27 ± 2.1 n = 14	3 ± 5.3 n = 7	42 6.3 n = 7	31	119
Noradrenaline	Guanethidine	27 _ 2.1 n = 14	45 6.9 n = 7	43 8.6 n = 7	131	123
Angiotensin	Phenoxybenzamine	5 ± 1.8 n = 17	1 ± 6.9 n = 7	40 5.6 = 7	26	105
Angiotensin	Guanethidine	25 ± 1.8 n = 17	−2 7.0 n = 7	41 7.9 n = 7	23	112

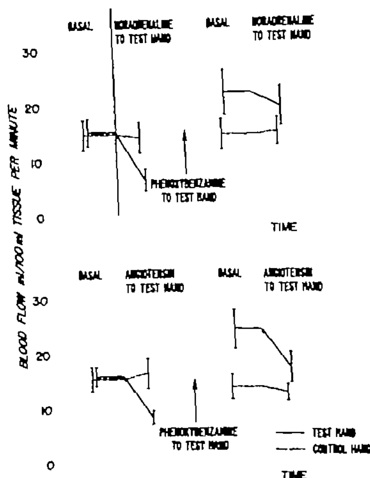


Fig. 3 Effect on blood flow in the hand of intra-arterial infusion of noradrenaline (mean of 5 experiments) and of angiotensin (mean of 7 experiments) into the brachial artery on the same side as the test hand (solid line) before and after administration of phenoxybenzamine into the same artery The other hand served as control (dashed line). Mean ± s.e.m.

shown in table 1 The increase in peripheral vascular resistance produced by noradrenaline was reduced by phenoxybenzamine, but not by guanethidine. However both vasodilator drugs inhibited to a large extent the effect of angiotensin on the peripheral vascular resistance.

E. *Effects of phenoxybenzamine on the action of intra-arterially administered noradrenaline and angiotensin in the hand*

The effects of intra-arterially administered noradrenaline and angiotensin on the blood flow in the hands before and after intra-arterial ad

ministration of phenoxybenzamine to the same hand (test hand) were studied in 5 and 7 experiments, respectively (fig. 3) After phenoxybenzamine the blood flow gradually increased in the test hand and reached a stable level in 30–60 minutes The increase in the blood flow after phenoxybenzamine was on an average about the same in the study with intra-arterially infused noradrenaline as in the studies reported in section B, while in the study with intra arterially angiotensin, the mean blood flow increase after phenoxybenzamine was more marked (fig. 3)

Before the administration of phenoxybenzamine, the *intra-arterial infusion of noradrenaline* (1/60 of the intravenous dose) reduced the blood flow in the test hand by 8 7 ± 1 62 ml. Only small and variable changes were observed in the control hand in which the blood flow decreased by 0 5 ± 1 11 ml. The "net effect" of noradrenaline can be calculated according to the formula *"Net effect"* = (*test minus control hand blood flow before the drug*) minus (*test minus control hand blood flow after the drug*) with this method of calculation a "net decrease" of 8.2 ± 1.86 ml ($p < 0.02$) after noradrenaline was found in the test hand. This decrease was of the same order of magnitude as observed when noradrenaline was infused intravenously *After phenoxybenzamine, noradrenaline reduced the blood* flow to a lesser extent and the "net effect" of noradrenaline was then 3.0 ± 0 84 ml ($p < 0.05$) The difference between the effect of noradrenaline on the blood flow before and after phenoxybenzamine was 5.2 ± 1 49 ml ($p < 0.05$)

The dose of the *intra-arterially administered angiotensin* was about 1/5 of that given intravenously By calculating the "net effect" of angiotensin, as described above for noradrenaline, an intra-arterial infusion was found to decrease the blood flow by 8 4 ± 1 15 ml ($p < 0.001$), and this decrease was similar to that observed after intravenous administration of the drug. After phenoxybenzamine the decrease in blood flow of angiotensin was of the same order of magnitude as before and the "net effect" was then a decrease of 6 1 ± 1 68 ml ($p < 0.02$). The difference between the effect of angiotensin on the blood flow before and after phenoxybenzamine was 2.3 ± 1 52 ml

Discussion

The blood flow in the hands is characterized by marked spontaneous variations and is greatly influenced by emotional stimuli (cf review by GREENFIELD 1963). Hence consideration must be given to the possibility that the intravenously administered noradrenaline or angiotensin was consciously noted by the subject, leading to increased attention, which in turn could be responsible for the increased vascular resistance in the

hand through a sympatho-adrenal discharge. This possibility seems to be excluded by the fact that the subjects were apparently completely unaware of the drug infusions and on questioning afterwards denied any sensations. The results of this study are in full agreement with those obtained in our previous study on the indirect effect of angiotensin in which the subjects were given a hypnotic dose of pentobarbitone to eliminate the influence of psychic factors (JOHNSSON, HENNING & ÅBLAD 1965).

The intra-arterial doses of phenoxybenzamine and guanethidine used in the present study have previously been found to bring about a partial block of the vasoconstrictor tone in the hand phenoxybenzamine acting through an alpha receptor block and guanethidine through an adrenergic neuron block (HENNING & JOHNSSON 1967).

The vasoconstrictor effect of intravenously or intra-arterially administered noradrenaline was antagonized by phenoxybenzamine. The effect of intravenously administered noradrenaline was, if anything, increased by guanethidine. These data are consistent with data previously reported (HENNING & JOHNSSON 1967) and indicate that both intravenously and intra-arterially administered noradrenaline elicit vasoconstriction in the hand through a direct stimulation of its vascular alpha receptors.

The vasoconstrictor effect of intravenously administered angiotensin differed from that of noradrenaline since both phenoxybenzamine and guanethidine produced a marked inhibition. The finding that intra arterially administered guanethidine inhibited the effect of intravenously infused angiotensin, indicates that the vasoconstrictor effect of angiotensin in the hand is mainly due to an effect mediated via the sympathetic vasoconstrictor fibres. If release of catecholamines from the adrenal medulla (cf FELDBERG & LEWIS 1964 ROBINSON 1965 PEACH, CLINE & WATTS 1966) had been a significant factor in the vasoconstrictor effect of angiotensin in this study the alpha-adrenergic receptor blocking agent phenoxybenzamine would probably have antagonized the effect more than the adrenergic neuron blocker guanethidine The reason why the effect of intravenously infused angiotensin was not completely abolished might depend on the fact that sympathetic activity was not totally blocked by the vasodilator drugs in the doses used (cf HENNING & JOHNSSON 1967) These results obtained with intravenously infused angiotensin, which indicate an effect via the sympathetic vasoconstrictor fibres, confirm those obtained in a previous study (JOHNSSON HENNING & ÅBLAD 1965), and are consistent with those of SCROOP & WHELAN (1966). These investigators observed that the vasoconstrictor effect of angiotensin in the hand was greatly reduced by phenoxybenzamine or bretylium tosylate and absent in a surgically sympathectomized hand.

The localization within the sympathetic nervous system of this effect of angiotensin is not known Possible sites of attack are e.g. the sympathetic centres in the brain or spinal cord, the sympathetic ganglia, the post ganglionic nerve endings or sympathetic reflex mechanisms. The results of our experiments with intravenously infused angiotensin indicate that this drug affects the sympathetic system though the exact site of action is unknown However a comparison between the results obtained with intra-arterial and intravenous infusions of noradrenaline and angiotensin suggests that the peripheral sympathetic nerve endings in these experiments were not greatly influenced by angiotensin. The ratio between the intra-arterial and intravenous dose of angiotensin, which produced similar decreases in blood flow in the hand was approximately 1 5 whereas that for noradrenaline was 1 60 These figures are of the same order of magnitude as those reported by SCROOP WALSH & WHELAN (1965). It is probable that during intra-arterial administration, considerably more angiotensin reached the sympathetic nerve endings than when the drug was given intravenously however the decrease in blood flow was about the same in both cases. This may indicate that intravenously infused angiotensin affected the sympathetic nervous system proximal to the peripheral nerve endings. These findings are in agreement with data previously reported by JOHNSSON HENNING & ÅBLAD (1965) and SCROOP & WHELAN (1966) The observation that the effect of intravenous but not that of intra-arterial angiotensin was greatly reduced by phenoxybenzamine, may indicate that the effect on the hand blood flow of intravenously but not intra-arterially administered angiotensin is to a great extent mediated via the sympathetic nervous system. This finding is also consistent with a site of action of angiotensin on the sympathetic nervous system proximal to the peripheral nerve endings. It is probable that the effect of intra-arterially infused angiotensin in these experiments was mainly due to a direct stimulation of vascular smooth muscle via a receptor other than the adrenergic alpha-receptor

Summary

Intravenously infused angiotensin (mean dose 2.2 µg/minute) and noradrenaline bitartrate (mean dose 18 µg/minute) elicited vasoconstriction in the hand. Phenoxybenzamine administered into a brachial artery greatly reduced the effect of angiotensin and noradrenaline in the corresponding hand while guanethidine, also given into a brachial artery inhibited only the effect of intravenously infused angiotensin, but not that of noradrenaline.

Angiotensin, when administered into a brachial artery had to be given in a relatively high dose as compared with the intravenous dose in order to produce about the same reduction of blood flow in the ipsilateral hand Thus the dose ratio between intra-arterially and intravenously administered angiotensin was 1 5 while the corresponding ratio for noradrenaline was 1 60 Moreover the effect of intra-arterially administered angiotensin on the hand blood flow was not inhibited by phenoxybenzamine. The findings indicate that angiotensin interacts with the sympathetic nervous system and that the site of action for this effect on the hand blood flow seems to be mainly localized to a site proximal to the peripheral sympathetic nerve terminals.

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Comparative Effects of Intra-arterially Administered Isoprenaline and Sodium Nitrite on Blood Flow and Volume of the Forearm

By

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Isoprenaline stimulates vascular β-receptors and induces a vasodilatation in various vascular beds with a concomitant rise in blood flow (GREEN & KEPCHAR 1959 AHLQUIST 1965). This drug appears to be a particularly potent dilator of resistance vessels i.e. mainly arterioles in skeletal muscle (see e.g. GINSBURG & COBBOLD 1960) Animal studies by FOLKOW (1960) and ABBOUD ECKSTEIN & ZIMMERMAN (1965) indicate that isoprenaline also dilates capacitance vessels (i.e. predominantly the veins, see FOLKOW 1960 MELLANDER 1960) in this tissue. The capacitance response, however was found to be weak compared to the simultaneously recorded dilatation of the resistance vessels.

In the present investigation an attempt was made to evaluate the effects of intra-arterially administered isoprenaline on the resistance and capacitance vessels in the human forearm. A plethysmographic technique was used. The effects of isoprenaline were compared to those of sodium nitrite, a drug, which has a small dilator effect on resistance vessels but a marked effect on capacitance vessels (see e.g. WILKINS, HAYNES & WEISS 1937 ABLAD 1963)

Methods

The experiments were performed on healthy students according to a procedure modified from that of ABLAD & JOHNSSON (1963). For infusion of the drugs thin polyethylene catheter was introduced into brachial artery at elbow level, as previously described (JOHNSSON 1967). Plethysmographs were applied to the upper part of both forearms, and filled with water maintained at constant temperature of 34° The plethysmographs ere of the type described by FOLKOW GRIMBY & THULESIUS (1958), but the expansion chamber was modified according to DAHN (1964).

The measurement of the *blood flow in the forearms* was made by venous occlusion

plethysmography as previously described (JOHNSSON 1967). Flow was determined simultaneously in both forearms once or twice a minute. Continuous measurements of the *changes in the volume of the two forearms* were made separately from the recordings of the blood flow. During this procedure, the arms were kept in the same position as during the periods of blood flow recording. The upper part of the forearms was at the level of the sternal angle and the water level in the expansion chamber of the plethysmographs was *about 10 cm higher than the upper surface of the forearms. It was considered desirable to* maintain isovolumetric conditions in the forearms before the drugs were given. This was accomplished by maintaining a constant pressure of 20–25 cm H_2O in the proximal occlusion cuffs during the volume measurements. Hand circulation was occluded during the period of forearm volume recording and throughout all drug infusions by maintaining a suprasystolic pressure in the distal cuffs.

Four main types of experiments were performed. In one series of experiments isoprenaline was infused into a brachial artery (test forearm side) for 7–8 minutes and its effects on forearm blood flow or volume followed. (A in the results). These experiments were performed in order to determine when the effect of isoprenaline on blood flow and volume reached a steady state level.

In a second type of experiment the effects of both isoprenaline and sodium nitrite on forearm blood flow and volume were compared in the same experiment (B in the results). The basal blood flow was followed for five minutes. Five minutes later the basal forearm volumes were recorded for 3–4 minutes. During the continuous recording of the volumes isoprenaline or sodium nitrite was infused intra-arterially. Isoprenaline was infused for 7 minutes and sodium nitrite for 9 minutes. During the last two minutes of the infusion of the drugs the continuous recording of forearm volumes was stopped, and instead the blood flow was registered. After the subject had rested for at least 30 minutes the same test procedure was repeated, now with the other drug. Isoprenaline was given as the first drug to 4 subjects and sodium nitrite to 3 subjects. In two subjects an additional experiment was carried out, in which isoprenaline and sodium nitrite were *given in the same manner as* described. In these latter experiments plethysmographs were not applied but, instead, the arterial blood pressure was measured with conventional auscultatory technique.

In a third series of experiments, simultaneous measurements were made of the changes in volume and *venous pressure in the forearm* before and during intra-arterial infusion of isoprenaline or sodium nitrite (C in the results). Into a subcutaneous vein near the elbow on the volar part of the test forearm, a "Rochester" plastic catheter was introduced (KURT, WRIGHT & JENKO 1958). This catheter was connected to an open vertical plastic tube or to an infusion bottle containing isotonic saline. The venous pressure was measured by subtracting the height of the fluid level in the expansion chamber of the plethysmograph from the height of the level of the fluid column in the plastic tube. Every value for the venous pressure is based on 3–5 measurements in each subject. Isotonic saline was *infused at a rate* of 0.5 ml/minute through the venous catheter when the venous pressure was not measured. Isoprenaline was given as the first drug to two subjects and sodium nitrite to the other two subjects. The interval between the infusions of the two drugs was at least 30 minutes.

In a fourth series of experiments isoprenaline or sodium nitrite was administered into a brachial artery before and after infusion of propranolol into the same artery (D in the results). The volume changes in the forearms were studied during this experimental procedure.

The drugs used were l-isoprenaline sulphate, sodium nitrite and racemic propranolol hydrochloride. The intra-arterial doses of l-isoprenaline sulphate ranged from 0.006 to 0.015 *µg/minute* and those of sodium nitrite from 0.32 to 0.80 mg/minute. The dose of propranolol hydrochloride administered into a brachial artery was 0.50 mg.

Results are reported as means ± standard errors of the means (s.e.m.). The statistical analysis was based on the t-test (Fisher 1958). The "net effect" of the drugs on test forearm blood flow was calculated according to the formula (1)

Net effect" = (test minus control forearm blood flow after the drug) minus (test minus control forearm blood flow before the drug)

The "net effect" of the drugs on the volume of the test forearm was calculated in the corresponding manner

Results

A. Effects of isoprenaline on blood flow and volume of the forearm

The effect of intra-arterially infused isoprenaline on blood flow was studied in 5 subjects. Fig. 1 A shows the result of one experiment. Isoprenaline induced an increase of the blood flow in the forearm on the side of infusion (test arm), but no significant change in the other forearm (control arm) A marked increase in blood flow occurred immediately after the start of the infusion and a steady state level was reached within 2 3 minutes. This time-effect pattern was reproduced in the other four experiments. When comparing the effect of isoprenaline on blood flow during the period 3–4 minutes after the start of the infusion with the pre-drug value, a "net increase" of 5 4 ± 0 89 ml/100 ml tissue/minute (in the following "ml") was recorded (n = 5). The corresponding mean value for the same 5 subjects 5–6 minutes after the start of the infusion was 5.3 ± 0.85 ml. There was thus no significant difference between the effects on blood flow during the periods 3–4 and 5–6 minutes after the start of the infusion of isoprenaline (difference 0 1 ± 0.17 ml)

The effect of isoprenaline on forearm volume was studied on four subjects. Isoprenaline increased the volume of the test forearm This effect set in immediately after the start of the infusion and reached a peak value about one minute later (fig. 1 B) When the blood flow response to isoprenaline was recorded, a peak value was generally also recorded at the corresponding time. After about 2 minutes the forearm volume had almost levelled off and thereafter only small changes in volume occurred during the drug infusion. No significant changes in the volume occurred in the control forearm. During the period 3–4 minutes after the start of the infusion the "net increase" of the volume in the test arm was 0.42 ± 0 06 ml/100 ml tissue and during the following 2-minute period, the corresponding value was 0.44 ± 0 07 ml/100 ml tissue (difference 0 02 ± 0.03 ml/100 ml tissue) The effects of isoprenaline both on blood flow and forearm volume disappeared within 15 minutes after termination of the infusion of the drug.

The results thus indicate that isoprenaline produced an increase in test arm volume essentially co-ordinated in time with the effect on test arm

plethysmography as previously described (JOHNSSON 1967). Flow was determined simultaneously in both forearms once or twice a minute. Continuous measurements of *changes in the volumes of the two forearms* were made separately from the recordings of blood flow. During this procedure, the arms were kept in the same position as during periods of blood flow recording. The upper part of the forearms was at the level of the sternal angle and the water level in the expansion chamber of the plethysmograph about 10 cm higher than the upper surface of the forearms. It was considered desirable to maintain isovolumetric conditions in the forearms before the drugs were given. This was accomplished by maintaining a constant pressure of 20–25 cm H_2O in the proximal occlusion cuffs during the volume measurements. Hand circulation was occluded during the period of forearm volume recording and throughout all drug infusions by maintaining *suprasystolic pressure in the distal cuffs.*

Four main types of experiments were performed. In one series of experiments isoprenaline was infused into a brachial artery (test forearm side) for 7–8 minutes and its effect on forearm blood flow or volume followed (A in the results). These experiments were performed in order to determine when the effect of isoprenaline on blood flow and volume reached a steady state level.

In a second type of experiment the effects of both isoprenaline and sodium nitrite on forearm blood flow and volume were compared in the same experiment (B in the results). The basal blood flow was followed for five minutes. Five minutes later the basal forearm volumes were recorded for 3–4 minutes. During the continuous recording of the volumes isoprenaline or sodium nitrite was infused intra-arterially. Isoprenaline was infused for 7 minutes and sodium nitrite for 9 minutes. During the last two minutes of the infusion of the drugs the continuous recording of forearm volumes was stopped, and instead the blood flow was registered. After the subject had rested for at least 30 minutes the same procedure was repeated, now with the other drug. Isoprenaline was given as the first drug to 4 subjects and sodium nitrite to 3 subjects. In two subjects an additional experiment was carried out, in which isoprenaline and sodium nitrite were given in the same manner as described. In these latter experiments plethysmographs were not applied but instead the arterial blood pressure was measured with conventional auscultatory technique.

In a third series of experiments, simultaneous measurements were made of the changes in volume and *venous pressure in the forearm* before and during intra-arterial infusion of isoprenaline or sodium nitrite (C in the results). Into a subcutaneous vein near the elbow on the volar part of the test forearm, a Rochester plastic catheter was introduced (KIRBY, WRIGHT & JENSEN o 1958). This catheter was connected to an open vertical plastic tube and an infusion bottle containing isotonic saline. The venous pressure was measured by tracing the height of the fluid level in the expansion chamber of the plethysmograph and the height of the level of the fluid column in the plastic tube. Every value for the venous pressure is based on 3–5 measurements in each subject. Isotonic saline was infused at a rate of 0.5 ml/minute through the venous catheter when the venous pressure was not measured. Isoprenaline was given as the first drug to two subjects and sodium nitrite to the other subjects. The interval between the infusions of the two drugs was at least 30 minutes.

In a fourth series of experiments isoprenaline or sodium nitrite was administered into a brachial artery before and after infusion of propranolol into the same artery (D in the results). The volume changes in the forearms were studied during this experimental procedure.

The drugs used were 1-isoprenaline sulphate, sodium nitrite and racemic propranolol hydrochloride. The intra-arterial doses of 1-isoprenaline sulphate ranged from 0.0 ... 0.015 μg/minute and those of sodium nitrite from 0.32 to 0.80 mg/minute. The dose of propranolol hydrochloride administered into a brachial artery was 0.50 mg.

Results are reported as means ± standard errors of the means (s.e.m.). The statistical analysis was based on the t-test (F[illegible] 19[illegible]). The "net effect" of the drugs on test forearm blood flow was calculated according to the formula (I):

Net effect = (*test minus control forearm blood flow after the drug*) *minus* (*test minus control forearm blood flow before the drug*)

The "net effect" of the drugs on the volume of the test forearm was calculated in the corresponding manner.

Results

A. *Effects of isoprenaline on blood flow and volume of the forearm*

The effect of intra-arterially infused isoprenaline on blood flow was studied in 5 subjects. Fig. 1A shows the result of one experiment. Isoprenaline induced an increase of the blood flow in the forearm on the side of infusion (test arm), but no significant change in the other forearm (control arm). A marked increase in blood flow occurred immediately after the start of the infusion and a steady state level was reached within 2-3 minutes. This time-effect pattern was reproduced in the other four experiments. When comparing the effect of isoprenaline on blood flow during the period 3–4 minutes after the start of the infusion with the pre-drug value, a "net increase" of 5.4 ± 0.89 ml/100 ml tissue/minute (in the following "ml") was recorded (n = 5). The corresponding mean value for the same 5 subjects 5–6 minutes after the start of the infusion was 5.3 ± 0.85 ml. There was thus no significant difference between the effects on blood flow during the periods 3–4 and 5–6 minutes after the start of the infusion of isoprenaline (difference 0.1 ± 0.17 ml).

The effect of isoprenaline on forearm volume was studied on four subjects. Isoprenaline increased the volume of the test forearm. This effect set in immediately after the start of the infusion and reached a peak value about one minute later (fig. 1B). When the blood flow response to isoprenaline was recorded, a peak value was generally also recorded at the corresponding time. After about 2 minutes the forearm volume had almost levelled off and thereafter only small changes in volume occurred during the drug infusion. No significant changes in the volume occurred in the control forearm. During the period 3–4 minutes after the start of the infusion the "net increase" of the volume in the test arm was 0.42 ± 0.06 ml/100 ml tissue and during the following 2-minute period, the corresponding value was 0.44 ± 0.07 ml/100 ml tissue (difference 0.02 ± 0.03 ml/100 ml tissue). The effects of isoprenaline both on blood flow and forearm volume disappeared within 15 minutes after termination of the infusion of the drug.

The results thus indicate that isoprenaline produced an increase in test arm volume essentially co-ordinated in time with the effect on test arm

plethysmography as previously described (JOHNSSON 1967). Flow was determined simultaneously in both forearms once or twice a minute. Continuous measurements of the *changes in the volume of the two forearms* were made separately from the recordings of the blood flow. During this procedure, the arms were kept in the same position as *during the* periods of blood flow recording. The upper part of the forearms was at the level of the sternal angle and the water level in the expansion chamber of the plethysmographs was about 10 cm higher than the upper surface of the forearms. It was considered desirable to maintain isovolumetric conditions in the forearms before the drugs were given. This was accomplished by maintaining a constant pressure of 20–25 cm H_2O in the proximal occlusion cuffs during the volume measurements. Hand circulation was occluded during the period of forearm volume recording and throughout all drug infusions by maintaining a suprasystolic pressure in the distal cuffs.

Four main types of experiments were performed. In one series of experiments isoprenaline was infused into a brachial artery (test forearm side) for 7–8 minutes and its effects on forearm blood flow or volume followed (A in the results). These experiments were performed in order to determine when the effect of isoprenaline on blood flow and volume reached a steady state level.

In a second type of experiment the effects of both isoprenaline and sodium nitrite on forearm blood flow and volume were compared in the same experiment (B in the results). The basal blood flow was followed for five minutes. Five minutes later the basal forearm volumes were recorded for 3–4 minutes. During the continuous recording of the volumes isoprenaline or sodium nitrite was infused intra-arterially. Isoprenaline was infused for 7 minutes and sodium nitrite for 9 minutes. During the last two minutes of the infusion of the drugs the continuous recording of forearm volumes was stopped, and instead the blood flow was registered. After the subject had rested for at least 30 min the same test procedure was repeated, now with the other drug. Isoprenaline was given as the first drug to 4 subjects and sodium nitrite to 3 subjects. In two subjects an additional experiment was carried out, in which isoprenaline and sodium nitrite were given in the same manner as described. In these latter experiments plethysmographs were not applied but instead, the arterial blood pressure was measured with conventional auscultatory technique.

In a third series of experiments, simultaneous measurements were made of the changes in volume and *venous pressure in the forearm* before and during intra-arterial infusion of isoprenaline or sodium nitrite (C in the results). Into a subcutaneous vein near the elbow on the volar part of the test forearm a "Rochester" plastic catheter was introduced (KURTZ, WRIGHT & JUNKO 1958). This catheter was connected to an open vertical plastic tube or to an infusion bottle containing isotonic saline. The venous pressure was measured by subtracting the height of the fluid level in the expansion chamber of the plethysmograph from the height of the level of the fluid column in the plastic tube. Every value for the venous pressure is based on 3–5 measurements in each subject. Isotonic saline was infused at a rate of 0.5 ml/minute through the venous catheter when the venous pressure was not measured. Isoprenaline was given as the first drug to two subjects and sodium nitrite to the other two subjects. The interval between the infusions of the two drugs was at least 30 minutes.

In a fourth series of experiments isoprenaline or sodium nitrite was administered into a brachial artery before and after infusion of propranolol into the same artery (D in the *results*). The volume changes in the forearms were studied during this experimental procedure.

The drugs used were l-isoprenaline sulphate, sodium nitrite and racemic propranolol hydrochloride. The intra-arterial doses of l-isoprenaline sulphate ranged from 0.006 to 0.015 μg/minute and those of sodium nitrite from 0.32 to 0.80 mg/minute. The dose of propranolol hydrochloride administered into the brachial artery was 0.50 mg.

Results are reported as means ± standard errors of the means (s.e.m.). The statistical analysis was based on the t-test (FISHER 1958). The "net effect" of the drugs on test forearm blood flow was calculated according to the formula (I)

Net effect" = (test minus control forearm blood flow after th drug) minus (test minus control forearm blood flow before the drug)

The "net effect of the drugs on the volume of the test forearm was calculated I the corresponding manner

Results

A. *Effects of isoprenaline on blood flow and volume of the forearm*

The effect of intra-arterially infused isoprenaline on blood flow was studied in 5 subjects. Fig. 1 A shows the result of one experiment. Isoprenaline induced an increase of the blood flow in the forearm on the side of infusion (test arm), but no significant change in the other forearm (control arm) A marked increase in blood flow occurred immediately after the start of the infusion and a steady state level was reached within 2 3 minutes. This time-effect pattern was reproduced in the other four experiments. When comparing the effect of isoprenaline on blood flow during the period 3–4 minutes after the start of the infusion with the pre drug value, a "net increase" of 5 4 ± 0.89 ml/100 ml tissue/minute (in the following "ml") was recorded (n = 5). The corresponding mean value for the same 5 subjects 5–6 minutes after the start of the infusion was 5.3 ± 0.85 ml There was thus no significant difference between the effects on blood flow during the periods 3–4 and 5–6 minutes after the start of the infusion of isoprenaline (difference 0 1 ± 0 17 ml).

The effect of isoprenaline on forearm volume was studied on four subjects. Isoprenaline increased the volume of the test forearm. This effect set in immediately after the start of the infusion and reached a peak value about one minute later (fig. 1 B) When the blood flow response to isoprenaline was recorded, a peak value was generally also recorded at the corresponding time After about 2 minutes the forearm volume had almost levelled off and thereafter only small changes in volume occurred during the drug infusion. No significant changes in the volume occurred in the control forearm During the period 3–4 minutes after the start of the infusion the "net increase" of the volume in the test arm was 0.42 ± 0.06 ml/100 ml tissue and during the following 2-minute period the corresponding value was 0 44 ± 0 07 ml/100 ml tissue (difference 0.02 ± 0.03 ml/100 ml tissue) The effects of isoprenaline both on blood flow and forearm volume disappeared within 15 minutes after termination of the infusion of the drug.

The results thus indicate that isoprenaline produced an increase in test arm volume essentially co-ordinated in time with the effect on test arm

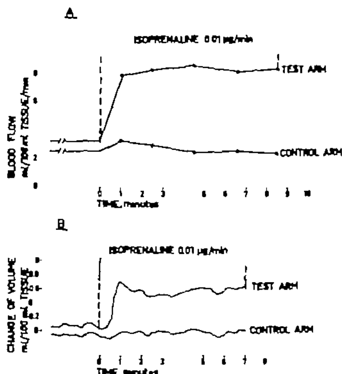


Fig 1 Effect of intra-arterial infusion of isoprenaline on forearm blood flow (A) and on forearm volume (B).

blood flow Furthermore, it seemed possible to compare the effects of isoprenaline on forearm blood volume 3–4 minutes after the start of the infusion of the drug with the forearm blood flow during the following 2 minute period since the circulatory effects of the drug were relatively constant during these periods.

B *Quantitative comparison between the effects of isoprenaline and sodium nitrite on blood flow and volume of the forearm*

In a previous investigation (Åblad & Johnsson 1963) it was shown that the effect of sodium nitrite on test forearm blood flow was well co-ordinated in time with its effect on forearm volume The effects of sodium nitrite on blood flow and volume set in immediately after the start of the infusion and the effects tended to level off within 5 minutes When the infusion was stopped the test arm blood flow and volume began to decrease and the blood flow reached the pre infusion value about 30 minutes later

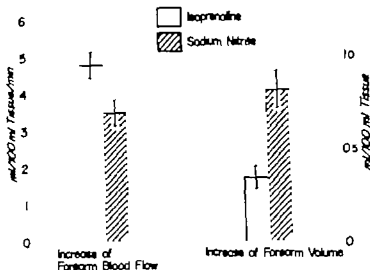


Fig 2. Increases of test forearm blood flow and olume during infusion f isoprenaline or sodium nitrite into the corresponding brachial artery Mea ± s.e.m (= 7)

The results reported above for isoprenaline and sodium nitrite indicated that it should be possible to compare the effects of both isoprenaline and sodium nitrite on the blood flow as well as on the volume of the forearm, in the same subject. This was done on seven volunteers. The results are presented in table 1 and figure 2.

Isoprenaline increased the blood flow in the test forearm by 4 7 ± 0 74 ml in 3-4 minutes after the start of the infusion of the drug No significant changes occurred in the control forearm (+0 1 ± 0.09 ml) The "net increase calculated according to Formula I was 4 6 ± 0 70 ml. The increase of the test forearm volume was 0 42 ± 0 09 ml/100 ml tissue, 5–6 minutes after the start of the infusion as compared to the pre-drug value. Only minor changes occurred in the control forearm (+0.07 ± 0 03 ml/100 ml tissue) and the "net increase" of the volume was 0.35 ± 0 06 ml/100 ml tissue.

After sodium nitrite the blood flow in the test forearm increased by 3.2 ± 0 74 ml, 5–6 minutes after the start of the infusion. No insignificant changes occurred in the control forearm (−0.2 ± 0 22 ml) The "net increase of the forearm blood flow was 3.4 ± 0 73 ml The increase of the test forearm volume was pronounced and amounted to 0 87 ± 0 10 ml/100 ml tissue, 7–8 minutes after start of the drug infusion. The volume of the control forearm did not change significantly (+0 05 ± 0 06 ml/100 ml tissue. The "net increase" was 0 82 ± 0.10 ml/100 ml tissue.

The arterial blood pressure – recorded in studies on two subjects – was

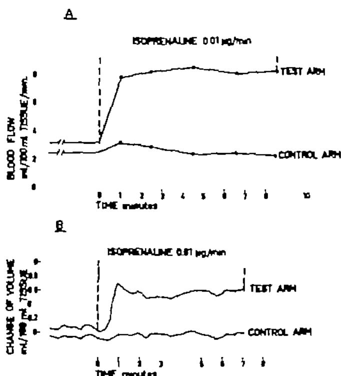
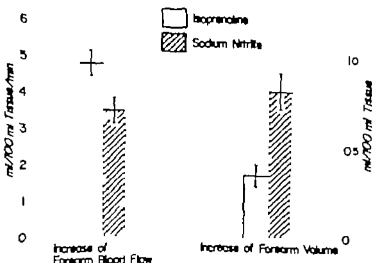


Fig. 1 Effect of intra-arterial infusion of isoprenaline on forearm blood flow (A) and on forearm volume (B).

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B *Quantitative comparison between the effects of isoprenaline and sodium nitrite on blood flow and volume of the forearm*

In a previous investigation (Åblad & Johnsson 1963) it was shown that the effect of sodium nitrite on test forearm blood flow was well co-ordinated in time with its effect on forearm volume. The effects of sodium nitrite on blood flow and volume set in immediately after the start of the infusion and the effects tended to level off within 5 minutes. When the infusion was stopped the test arm blood flow and volume began to decrease and the blood flow reached the pre-infusion value about 30 minutes later



F g. 2. Increases of test forearm blood flow nd volume during infusion of isoprenaline or sodium nitrite int the corresponding brachial artery Mea ± s.e.m. (— 7).

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Isoprenaline increased the blood flow in the test forearm by 4 7 ± 0 74 ml in 3-4 minutes after the start of the infusion of the drug. No significant changes occurred in the control forearm (+0.1 ± 0 09 ml) The "net increase" calculated according to Formula I was 4 6 ± 0.70 ml. The increase of the test forearm volume was 0 42 ± 0 09 ml/100 ml tissue, 5–6 minutes after the start of the infusion as compared to the pre-drug value. Only minor changes occurred in the control forearm (+0 07 ± 0 03 ml/100 ml tissue) and the "net increase" of the volume was 0.35 ± 0 06 ml/100 ml tissue

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Table 1

Effects of intra-arterial infusion of isoprenaline or sodium nitrite in the same experiment on blood flow and volume of the forearms. The drugs ere infused into the brachi l rtery of the test forearm. Blood flow in ml/100 ml tissue/minute. Volume change in ml/100 ml tissue
Mean ± s.e.m. (n = 7).

Drug Dose	Blood flow before the drug		Blood flow after the drug		Volume change after the drug	
	Test arm	Control rm	Test arm	Control arm	Test arm	Control arm
Isoprenaline 0.010 μg/min. (range 0.006–0.015)	3.5 0.54	3.3 ± 0.48	8.2 1.04	3.4 ± 0.51	0.42 ± 0.09	0.07 ± 0.03
Sodium nitrite 0.58 mg/min. (range 0.48–0.72)	3.5 ± 0.48	3.5 ± 0.49	6.7 ± 1.05	3.3 ± 0.40	0.87 ± 0.10	0.05 ± 0.06

not significantly influenced by the doses of isoprenaline or sodium nitrite administered intra-arterially

When compared in the same subjects, isoprenaline increased the blood flow more than sodium nitrite though the latter drug had a more marked effect on the forearm volume. Statistical evaluation of the differences between the calculated "net effects" within each experiment shows that the volume increase after isoprenaline was 0 47 ± 0.07 ml/100 ml tissue smaller than after sodium nitrite ($p < 0.001$) and that the average increase in blood flow was 1.2 ± 0.51 ml greater after isoprenaline than after sodium nitrite

For a further evaluation of the results a calculation was made of the quotient

$$\frac{\text{"net increase" of test forearm blood flow}}{\text{"net increase" of test forearm volume}}$$

In the seven experiments the quotient for isoprenaline was 14 8 ± 2.5 and for sodium nitrite 4 7 ± 1 1 The difference between the two quotients was 10.1 ± 2.4 ($p < 0\ 01$).

C. *Quantitative comparison between the effects of isoprenaline and sodium nitrite on volume and venous pressure of the forearm*

In four subjects the volume changes in the forearms after isoprenaline or sodium nitrite were recorded simultaneously with the subcutaneous venous pressure in the test forearm (fig. 3). The intra-arterial mean dose of isoprenaline (0 013 µg/minute) was somewhat higher and that of sodium nitrite (0 51 mg/minute) somewhat lower than in the studies reported under B (table 1)

Before infusion of isoprenaline the venous pressure was *9 1 ± 1.03* cm H_2O When isoprenaline was administered intra arterially into the test forearm, the venous pressure increased and this change followed a similar time course as the volume increase. When the effect of isoprenaline was relatively stable, the venous pressure had increased to 10.2 ± 0.91 cm H_2O (increase 1 1 ± 0 13 cm H_2O) 3–4 minutes after the start of the infusion of isoprenaline. The "net increase" of the test forearm volume was 0.44 ± 0.06 ml/100 *ml* tissue during the same 2 minute period.

Before the administration of sodium nitrite the subcutaneous venous pressure was 8 6 ± 0.90 cm H_2O in the test forearm. During the infusion of sodium nitrite, the venous pressure increased somewhat and this change followed a similar time course as the volume increase. When the effect of sodium nitrite had reached a steady level, the venous pressure was ± 0.83 cm H_2O (increase 0.5 ± 0 13 cm H_2O), while the "net

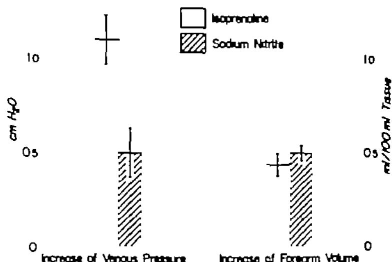


Fig. 3 Increases of venous pressure and volume in the test forearm during infusion of isoprenaline or sodium nitrite into the corresponding brachial artery Mean ± s.e.m. (n = 4).

of the test forearm volume was 0 50 ± 0 04 ml/100 ml tissue during the same period (5–6 minutes after the start of the infusion of sodium nitrite).

In every experiment isoprenaline produced a greater increase in venous pressure than sodium nitrite provided the two drugs were compared at a time when both drugs had produced a similar increase of the test forearm volume. Statistical evaluation of the difference between the calculated effects within each experiment shows that the increase of the venous pressure was 0 6 ± 0.10 cm H_2O ($p < 0\,01$) higher after isoprenaline than after sodium nitrite, while the volume increase was, if anything, somewhat higher after sodium nitrite (difference 0 07 ± 0 04 ml/100 ml tissue)

D *Effect of propranolol on isoprenaline-induced increase of forearm volume*

The effect of propranolol on the isoprenaline-induced increase of the forearm volume was studied in three subjects. Before the propranolol infusion, intra arterially administered isoprenaline produced a "net increase" of the test arm volume of 0 45 ± 0 11 ml/100 ml tissue Propranolol (0 50 mg into the brachial artery) totally abolished the effect of isoprenaline on the forearm volume ("net change" −0 01 ± 0.05 ml/100 ml tissue). In an additional experiment it was found that propranolol did not influence the volume increase due to sodium nitrite

Discussion

The results show that isoprenaline dilated the resistance vessels relatively more than sodium nitrite, but increased the volume of the forearm relatively less than sodium nitrite.

The increase in the volume of the forearm produced by the two drugs could be the result of changes in vascular blood capacity or of changes in extravascular tissue volume secondary to changes in transcapillary fluid exchange. The latter changes could be due to an altered capillary mean hydrostatic pressure brought about by a change in the ratio of the pre-to postcapillary resistance (MELLANDER 1960). The effects of vasodilator drugs on transcapillary fluid exchange can be evaluated from the tissue volume changes under steady state vasoconstriction or vasodilatation (MELLANDER 1960). In the present experiments isoprenaline induced a very rapid increase of both the volume and the blood flow of the forearm. The blood flow was essentially unchanged and the forearm volume tended to increase only slightly from 2–3 minutes after the start of the infusion until the infusion was terminated. Thus the predominant part of the forearm volume increase after isoprenaline appears to be due to an increased vascular capacity. The increase in forearm volume during infusion of sodium nitrite intra-arterially was probably also mainly due to an increased vascular capacity since the volume increase has been found to level off at the same time as the blood flow reaches a steady state, i.e. about five minutes after the start of the infusion of the drug (ÅBLAD & JOHNSSON 1963). In studies on cats ÅBLAD & MELLANDER (1963) showed that the vasodilator effect of sodium nitrite was not accompanied by any significant change of extravascular fluid volume.

Isoprenaline could have increased the vascular blood capacity in the forearm in two ways. The increase could be partly explained by an "active" reduction of the smooth muscle tone in the capacitance vessels and partly by an increase due to a passive distension of the post-arteriolar vessels, since the pressure in these vessels could be expected to rise secondary to the dilatation of the resistance vessels. Isoprenaline produced a comparatively greater reduction in vascular resistance and increased venous pressure relatively more than did sodium nitrite. Hence, it can be concluded that the passive distention of post-arteriolar capacitance vessels should be of relatively greater importance for the volume increase due to isoprenaline, than for that due to sodium nitrite. It can not be excluded that the greater part of the increased vascular capacity produced by isoprenaline was due to a passive distension of capacitance vessels.

In view of these considerations it can be concluded that the "active"

vasodilator response of isoprenaline was relatively more marked on the resistance vessels, while that of sodium nitrite was comparatively more marked on the capacitance vessels.

In order to elucidate whether isoprenaline has any "active" dilating effect on capacitance vessels, a study was performed on anaesthetized cats (JOHNSSON & ÖBERG 1967) The responses of the resistance and capacitance vessels in the cat hindquarter to isoprenaline, nitroglycerin and sodium nitrite were recorded. The results obtained in this animal study agreed well with those in man in that isoprenaline produced a greater dilatation in resistance vessels, but a smaller increase in total volume, than either sodium nitrite or nitroglycerin. To elucidate the magnitude of the "active" dilating effects on the capacitance vessels, drug infusions were also performed while the blood flow in the hindquarter was maintained constant The venous transmural pressure can then be expected to be relatively constant during the infusion period. Volume changes will, in that case, only reflect an "active" dilating effect by the drugs on the capacitance vessels. The results indicate that isoprenaline had a small "active" dilating effect on the capacitance vessels, while the effects produced by sodium nitrite and nitroglycerin were considerably greater

Previous investigations have indicated that the effects of different drugs on consecutive vascular beds in man agree well with those in cats (cf FOLKOW 1960 ÅBLAD & JOHNSSON 1963 ÅBLAD & MELLANDER 1963) The results in the present study are in agreement with the corresponding results in the study on cats by JOHNSSON & ÖBERG (1967). Hence, it seems reasonable to assume that in man, too isoprenaline has a small "active" dilating effect on the capacitance vessels in a skin-skeletal muscle region. Such an interpretation is supported by the findings of SCHARPEY SCHAFER and GINSBURG (1962) By using a technique in which they related the rate of venous pressure rise per unit of inflow they showed that isoprenaline caused some venous dilatation in the forearm

The β-adrenergic blocking drug propranolol abolished the capacitance vessel response to isoprenaline in the present study The same dose of propranolol has been shown to produce almost total block of the response of the resistance vessel to isoprenaline in the forearm (JOHNSSON 1967). It has previously been reported by other investigators that isoprenaline-induced increase in forearm or leg blood flow is inhibited by β-adrenergic receptor antagonists (ABBOUD, ECKSTEIN & ZIMMERMAN 1964 LOWE & ROBINSON 1964 BRICK *et al* 1966 MAHON 1966 SCHOOP & SCHMIDTKE 1966). These findings are in agreement with Ahlquist s postulate that the effects of isoprenaline are primarily due to activation of β-adrenergic receptors (AHLQUIST 1948)

The present results indicate that in skin-skeletal muscle tissue vasodila

tation through β-receptor activation is of greater importance for the control of the resistance function than of capacitance function. FOLKOW (1960) has previously come to the same conclusion on the basis of studies with isoprenaline in animals and with adrenaline in man

Summary

Isoprenaline (0 010 μg/min.) or sodium nitrite (0 58 mg/min.) was infused into a brachial artery and the effects on blood flow and volume of the upper part of the forearm were studied separately Both drugs increased the forearm blood flow and volume. A comparison of the effects of the two drugs showed that isoprenaline produced a greater increase in blood flow but a considerably smaller increase in volume of the forearm than did sodium nitrite. The relative importance of various mechanisms for the volume increase is discussed.

On the basis of the present results and results of animal studies, it is concluded that isoprenaline has a marked spasmolytic effect on the resistance vessels and a weak effect on capacitance vessels in a skin-skeletal muscle region. Sodium nitrite in relation to its effects on the resistance vessels, has a considerably more marked spasmolytic effect than isoprenaline on the capacitance vessels.

The volume increase produced by isoprenaline was completely blocked by propranolol (0.50 mg intra-arterially).

Acknowledgements

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Studies on the Metabolism of Chlorprothixene (Truxal®) in Rats and Dogs

By

I. Huus and A. Rauf Khan

(Received August 3, 1966)

A good deal of work has been done to investigate the metabolic fate of phenothiazine tranquillizers and chlorpromazine. Up-to-date the data available on this work show sulphoxidation, ring hydroxylation and side chain degradation of chlorpromazine which has brought the number of identified metabolites of this compound to about fifteen (Fishman et al. 1965).

Chlorprothixene, trans-2-chloro-9-(3-dimethylaminopropylidene)-thioxanthene), has been in use as a tranquillizer for many years but no specific work has been published about its metabolic fate. Early investigations on this subject carried out in our laboratories, show the presence of chlorprothixene-sulphoxide in human and animal urine (Allgén et al. 1960). Improved methods of thin-layer chromatography have enabled us to study the biotransformation of this compound in animals in more detail and a general survey on this subject is reported here (fig. 1).

We have been unable to find as large a number of metabolites of this compound, as reported for chlorpromazine. This may be due to the fact that chlorprothixene contains a thiaxanthene instead of a phenothiazine ring. The excretion of chlorprothixene (CPT) also demonstrates its stability.

Methods

T dogs were used in the test. Urine was collected before treatment and used as control. A dose of 5 mg/kg of chlorprothixene was given intramuscularly for four days and 24 hours urine specimens were collected for the following six days. The urine samples were stored in the refrigerator and pooled together before analysis.

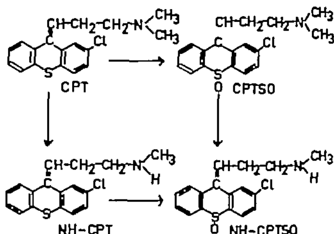


Fig. 1 Structure of identified metabolites of chlorprothixene (CPT): Chlorprothixene-sulphoxide (CPTSO) demethyl-chlorprothixene (NH-CPT) and demethyl-chlorprothixene-sulphoxide (NH-CPTSO)

Two groups of 10 rats were used in another test. Control urine was collected before medication. The rats were given a dose of 25 mg/kg intraperitoneally for four days. 24 hours urine samples were collected for the following six d ys and stored in the refrigerator

Extraction

For extraction, 500 ml of dog's urine was concentrated to 100 ml in a rotary ev porator at 40° under vacuum and 50 ml of rat s urine was taken for extraction. The urine as adjusted to pH 9.0 by addition of 30% sodium hydroxide solution and extracted with two equal vol mes of ethylene-dichloride To the residual rine from the first extractions, 50 sodium hydroxide sol tion equal to 1/10 of it volume was added and again extracted ith two equal volumes of ether (this procedure was dopted to extract ll possible polar and non-polar metabolites).

The urine was shaken mechanically for 30 minutes nd separated by centrifuging the contents fo 10 minutes at 10.000 /m. The separated organic extracts were dried over anhydrous sodium ulphate and filtered. The filtrate was dried in rotary evaporator under vacu m. The residue was dissolved in 0.5 ml of ethanol in the case of the dog's urine and in 0.3 ml of ethanol in the case of rat urine.

Hydrolysis

The residual rine after the xtractions, was subjected to acuum at 40 for few minutes to remove any traces of organic solvent and then adjusted to pH 5.5 by the addition of 6 N hydrochloric cid. The urine samples were divided int t portions. To one portion β-glucuronidase (bacterial) w added to give a concentration of 500 nits/ml of urine and incubated for 40 hours t 37 while the other portion was incubated for the same period without the addition of β-glucuronidase The urine was then adjusted to pH 9.0 by adding 1 N sodium hydroxide solution, extracted with equal olumes of ethylene-dichloride and prepared for chromat graphy as mentioned above.

Chromatography

250 μ thick plates f Kieselgel G nach Stahl (Merck) were prepared and activated at 110° for one hour 10–30 μl of ethanolic extract was spotted on the plates and developed in the following three solvent systems.

1 Benzene Ethylacet te Diethylamine (70 20 10).
2 Chloroform Acet Diethylamine (87 3 10).
3 Benzene Isopropanol Ammonia 1 / (45 35 20) (upper phase used).

For UV examination of the chromatogram, the plates were sprayed with mixture f sulphuric acid formaline (48 2). Other locating reagents such as Dragend rff reagent (STAHL 1965) and Iodoplatinate reagent (SMITH 1963) were also used. For identification of the spots, ninhydrin (f primary mines) nitroprusside-acetaldehyde (for secondary amines) (FEIGMAN & GOLDENBERG 1960) ferric chloride methanolic, ferric chloride-potassium ferricyanide and fast blue salt B (S AHL 1960) (for detection of phenols) were used. Persulphate reagent (SMITH 1960) was used to differentiate the spots. For UV bsorption studies, the corresponding spots were eluted in 0.1 N-HCl from duplicate unstained chromatogram and bsorption was read Zeiss PMQ II spectrophotometer using eluate of an equal area of the same plate as blank.

Results and Discussion

A) *Non-conjugated fraction of urine*

In urine from rats and dogs various spots detected on a thin-layer chromatogram (fig. 2) are shown in table 1 and 2 and which also give their approximate R_F-values in three solvent systems.

Spot 1 was considered to be actual chlorprothixene as indicated by its R_F-value in three solvent systems, identical to CPT It gave a strong yellow fluorescence in UV light when sprayed with a mixture of sulphuric acid-formaline and a pink colour with persulphate reagent. It gave a negative colour reaction with ninhydrin and nitroprusside-acetaldehyde

Table I

Approximate R_F-values of metabolites i the non-conjugated fraction of rats.

No.	Solvent system 1	Solvent system 2	Solvent system 3
1	85	.89	.85
2	77	.83	75
3	68	74	.64
4	.60	71	.54
5	.52	42	.35
6	44	.66	..5

Table 2

Approximate R_F-values of metabolites i the non-conjugated fraction of urine from dogs

No.	Solvent system 1	Solvent system	Solvent system 3
1	.85	.89	.83
2	77	.84	75
3	63	70	.64
4	–	–	
5	–	–	
6	44	.66	.25

indicating that demethylation of the side chain had not occurred. The corresponding spot when eluted in 0 1 N HCl from a duplicate unstained chromatogram gave peak absorption at 268 and 230 nm similar to CPT (fig. 3)

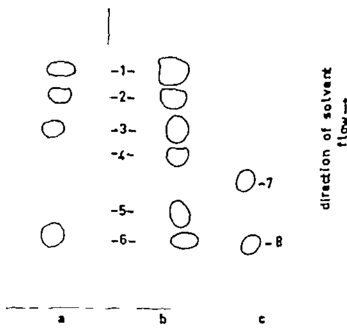


Fig – Chromatograms of alcoholic extract of dog's and rat s urine (non-conjugated and conjugated fraction), sol ent system Benzene-isopropanol mmonia I (45 35 70)

(a) Non-conjugated fraction of dog s urine

(b) Non-conj gated fraction f rat's urine

(c) C jugated fraction f d g urine.

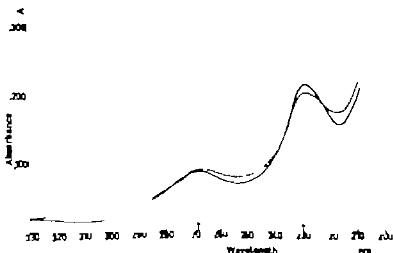


Fig. 3 UV absorptio curve for chlorprothixene 'CPT' (————)
UV absorption curve fo spot No. 1 'CPT' (– – – –)

Further evidence was available when the eluate of this spot in alcohol showed identical R_F-value and colour reaction with authentic chlorprotixene-sulphoxide (CPTSO) after oxidation with hydrogen peroxide (3 / alcoholic) This confirmed the excretion of CPT in urine by both dogs and rats. The identical R_F-value of spot No 2 to the authentic CPTSO suggested that it was CPTSO The spot gave a distinct red fluorescence with sulphuric acid-formaline mixture and a blue fluorescence in UV with persulphate reagent but a negative colour reaction with ninhydrin and nitroprusside-acetaldehyde reagent, showing that it was the simple oxidation of the ring sulphur The spot when eluted from duplicate unstained chromatogram in 0 1 N HCl for UV absorption studies gave a peak absorption at 261 and 221 nm similar to CPTSO (fig. 4).

Spot No 6 was considered to be monodemethylchlorprothixene-sulphoxide (NH-CPTSO) as it gave identical R_F-values in three solvent systems and a reaction with persulphate reagent (blue fluorescence in UV) similar to the authentic NH-CPTSO synthesized in our laboratories. A negative colour reaction with ninhydrin and a positive colour reaction with nitroprusside-acetaldehyde (blue) confirmed the presence of a monodemethylated side chain. The spot was eluted from a duplicate unstained chromatogram in 0 1 N HCl and showed in UV-absorption peak absorption at maximum 261 and 221 nm typical of sulphoxide (fig. 5).

Neither of the two identified metabolites i.e CPTSO and NH-

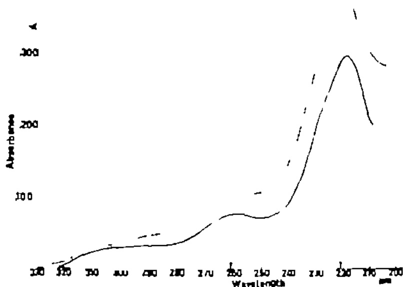


Fig. 4. UV absorption curve for authentic chlorprothixene-sulfoxide 'CPTSO' (———)
UV absorption curve for spot No. 2 CPTSO (– – – –)

was a sulphone as in that case the peak absorption in UV would have shifted from 221 to 205 nm.

None of the spots shown in table 1 and 2 gave positive colour reaction

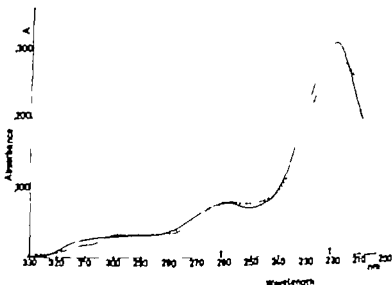


Fig. 5 UV absorption curve for authentic monodemethyl-chlorprothixene-sulfoxide 'NH CPTSO (———)
UV absorption curve for spot No. 6 'NH-CPTSO' (– – – –)

with ferric chloride (methanolic) ferric chloride-potassium ferricyanide or with fast blue salt B thus indicating the absence of phenol types of metabolites. It is possible that NH-CPT is excreted by dogs and rats and spot No. 3 was considered to be the same, as its R_F-value in three solvent systems was practically identical with the authentic NH-CPT But its colour reaction with a mixture of sulphuric acid formaline (reddish pink fluorescence) did not correspond to the colour reaction of NH-CPT (yellow fluorescence) This may be due to the fact that oxidation has taken place at the N position. Only slight amounts of the other two metabolites were found in the non-conjugated fraction of rat s urine and they showed an orange fluorescence in UV light after spraying with sulphuric acid formaline mixture.

B) *Conjugated fraction of urine*

In the urine from rats after hydrolysis for 40 hours with β-glucuronidase at pH 5.5 and subsequent extraction at pH 9 0 with ethylene-dichloride, we have not been able to detect any spot giving a colour reaction for phenols as mentioned above. This shows that ring hydroxilation had not occurred. In the case of the glucuronide fraction of dog s urine we have been able to detect two spots which gave a faint yellow colour with methanolic ferric chloride and a distinct yellow colour with fast blue salt B

After the spraying of fast blue salt B and when the same chromatogram was oversprayed with a mixture of sulphuric acid-formaline, the two spots mentioned above gave a bright reddish violet colour which indicated that the spots represented two metabolites. Their approximate R_F-values in three solvent systems are shown in table 3 (conf. fig. 2c).

As we have not been able to synthesize the phenol type of authentic metabolites, the comparison of colour reaction obtained and their identification have to be deferred until we can get some phenolic types of metabolites of CPT

Summary

The metabolic fate of chlorprothixene has been studied by thin-layer chromatographic methods. The excretion of unchanged chlorprothixene along with two of its metabolites i.e. chlorprothixene-sulphoxide (CPTSO) and monodemethyl-chlorprothixene-sulphoxide (NH-CPTSO) by dogs and rats is described. Hydroxilation of the thiaxanthene ring by dogs is also indicated but the identification of phenolic type of metabolites cannot be undertaken at present since authentic metabolites of this type are not available.

Table 3

Approximate R_F-values of metabolites in the conjugated fraction of urine from dogs.

No.	Solvent system 1	Solvent system 2	Solvent system 3
7	47	67	.46
8	.34	47	[illegible]

Approximate R_F-values of standards when taken from urine:

	Solvent system 1	Solvent system 2	Solvent system 3
CPT	85	.89	.85
CPTSO	77	.84	75
NH-CPT	.64	74	65
NH-CPTSO	44	.66	.25

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Comparison of the Inhibitory Effects of Tertiary and Quaternary Compounds on the Gastric Secretion of Rats

By

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In a previous study (ANTONSEN & NIELSEN 1963) in which the effects of atropine and a quaternary atropine compound were compared, it was found that the quaternary compound had a more marked and prolonged effect on the gastric secretion.

In order to determine to what extent the quaternary structure as such has an influence on the inhibitory effect on the stomach, we compared some new quaternary compounds with the corresponding tertiary analogues.

The compounds tested included one with a strong anticholinergic action and some with an inhibitory effect on the stomach, but no anti cholinergic or other spasmolytic actions.

The absorptions of quaternary and tertiary compounds from the gastro-intestinal canal differed appreciably and it was necessary to test their actions after both oral and parenteral administration.

Experimental Methods

The spasmolytic effects were tested the isolated guinea-pig intestine, as described previously (ANTONSEN & NIELSEN 1963).

The effects on the stomach were tested partly on Shay rats in the same manner as previously described (ANTONSEN 1953 ANTONSEN & NIELSEN 1963 ANTONSEN 1965a), and partly according t modification of THORNTON & CLIFTON's (1959) test-meal method (ANTONSEN 1965b).

Compounds tested

Two quaternary non-anticholinergic compounds, described previously (Antonsen 1965a) benzyl-tri-(β-propoxyethyl)-ammonium iodide (U247 51), and benzyl-tri-(β-butoxyethyl)-ammonium bromide (U247–73)

The corresponding tertiary compounds: N N-dipropoxyethyl benzylamine, HCl (U247–125) and N,N-dibutoxyethyl benzylamine HCl (U247-90). The quaternary anticholinergic compound 1 methyl-4-piperidyl cyclopentyl phenylacetate methoiodide (U247–96) and the corresponding tertiary compound 1-methyl-4-piperidyl cyclopentyl phenylacetate citrate (U247 95).

Further the quaternary anticholinergic compound (diphenylmethoxy)-ethyl-methyl-diethyl ammonium iodide (metropin ®) was used for comparison, as in previous studies.

All these compounds were prepared in our chemical research laboratory

In addition, atropine sulphate was included in some of the tests.

Results

Effect on isolated guinea-pig intestine

Neither U247–51 U247–73 nor the corresponding tertiary compounds U247–125 U247–90 had any antagonistic effect on acetylcholine, histamine, barium chloride and serotonin. When given in large doses they may produce some contraction of the intestine.

U247–95 was about 10 times more active than metropin ® and about 1 5 times more active than atropine as an anticholinergic drug. Its antihistaminic action was only about one-third of that of metropin ® whereas its antagonism of serotonin was 10 times stronger

U247–96 had an anticholinergic action which on pre-treatment for 30 seconds was the same as that of U247–95 namely 10 times more marked than that of metropin ® If the antagonists remains in the bath until full equilibrium has been attained its effect is 70 times that of metropin ® and about 7 times that of atropine. Its antagonism to histamine is no more than 1/30 and to serotonin about 10 times more than that of metropin ®

Effect on Shay rats

A. Anticholinergic compounds

The effects of intraperitoneal doses given simultaneously with ligation of the stomach are seen in table 1 The quaternary compound U247 96 had the strongest effect. The effect on the ulceration was 10 times greater than that of atropine, and the difference between the effects on the acid secretion was even greater The effect of U247–95 was likewise about 10 times that of atropine though smaller than that of U247–96 The duration of the mydriatic effect of atropine was similar to that of U247–96, but that of U247–95 was of considerably shorter duration

Table 1

Effects of metropin ®, U247-95, U247-96, and atropine administered intraperitoneally to Shay rats. Ligation period 9 hours. 4 animals to each dose.

Substance	Dose in mg	Ulcer index	Gastric contents: Volume ml	*pH*	Titration value in meq: pH 3.5	pH 9.2	Mydriatic activity: 15 min.	4–5 hrs.	9 hrs.
Control		1.0 × 100 % = 100	14.3	1.43	0.857	1.049	0	0	0
Metropin ®	2.0	0.4 × 80 % = 32	9.9	1.30	0.829	0.994	70 %	15 %	0
U247-95	0.25	0.4 × 50 % = 20	8.1	1.91	0.469	0.602	87 %	85 %	15 %
U247-96	0.5	0	2.9	2.07	0.127	0.232	100 %	100 %	95 %
Control		0.6 × 100 % = 60	12.9	1.68	0.757	0.932	0	0	0
Metropin ®	2.5	0	7.3	1.57	0.517	0.663	62 %	2 %	0
Atrop.sulph.	1.0	0.25 × 50 % = 12	10.7	1.56	0.929	1.084	95 %	85 %	70 %
U247-95	0.1	0.25 × 50 % = 12	11.1	1.58	0.756	0.895	65 %	20 %	%
U247-96	0.1	0	6.4	1.54	0.509	0.627	95 %	95 %	80 %
Control		0.6 × 100 = 60	14.8	1.43	0.962	1.204	0	0	0
Metropin ®	2.0	0.25 × 50 % = 12	7.5	1.38	0.528	0.668	55 %	13 %	0
Atrop.sulph.	1.0	0.25 × 50 % = 12	9.5	1.28	0.768	0.921	100 %	95 %	65 %
U247-96	0.1	0.11 × 50 % = 5	5.4	1.49	0.332	0.472	100 %	100 %	80 %

Table 2

Effects of metropin ® U247-95 U247-96, and atropine given by mouth to Shay rats 4 hours before the ligation Ligation period 16 hours. 4 animals to each dose.

Substance	Dose in mg	Ulcer index	Gastric contents: Volume ml	pH	Titration values in meq: pH 3.5	pH 9.2
Control		2.5 × 100 = 250	16.5	1.59	0.732	1.104
Metropin ®	10	4.0 × 100% = 400				
Atrop.sulph.	10	0.37 × 50 = 19	10.1	1.89	0.452	0.803
U247 95	10	1.75 × 75% = 130	11..	1.81	0.475	0.771
U247 96	10	0	8.2	2.01	0.294	0.598
Control		.75 × 100% = 275	14.2	1.63	0.535	0.825
U247 96	5	1.63 × 100% = 163	12.8	1.72	0.441	0.881
U 47 96	10	0.37 × 75% = 28	11..	1.88	0.424	0.791

Table 2 shows the effects of equal doses of these compounds given by mouth 10 mg metropin ® had no protective action. All the animals on this dose developed perforation of the stomach or oesophagus, so that figures for acid were not obtainable. The two tertiary compounds U247-95 and atropine had approximately the same effect on the acid secretion and atropine a slightly stronger effect on ulcers. U247-96 was found to have by far the strongest effect. Even if the compound is quaternary a sufficient amount is absorbed from the gastro-intestinal canal to give definite protection

Since the dose was given 4 hours before the experimental period, which extended over 16 hours, the duration of the effect of the test compound was very important. Atropine and U247-96 have prolonged effects and are bound firmly to the receptors, so that in the *in vitro* experiments on the intestine, for instance many washings were required to abolish the effect. Metropin ® and U247-95 on the other hand have relatively short, reversible effects.

The results set out in table 3 demonstrate the importance of prolonged action in experiments on Shay rats. The effect of metropin ® given in aqueous solutions was compared with the effect when given dissolved in 40 % polyvinyl pyrolidone (P.V.P.). P.V.P. reduces the absorption rate of a compound given subcutaneously (ANTONSEN & KROIGAARD 1953 ANTONSEN 1956). The figures for mydriatic effect show that P.V.P. prolonged

Table 3

Effect f metropin ® p ca in 40 solution of polyvinylpyrrolidone (P V P) to Shay rats Ligation period 9 hours. 5 animals t each dose

Dose of metropin ®	Ulcer index	Gastric contents				Mydriatic acti ity			
		V l me ml	pH	Titration alue l meq		15 min.	2 hrs.	5 hrs.	9 hrs.
				Co go	Phenolft.				
1 25 mg i p. in 0.9% N Cl	0.5 × 60% = 30	10.6	2.1	0 21	1.02	56%	16%	0	0
1.25 mg subc. i 0.9% N Cl	0.1 × 20% = 2	8.4	1 7	0 19	0.45	14%	85%	24%	0
1.25 mg subc. i 40% P V.P	0.2 × 20% = 4	6.7	2.8	0 02	0.17	10%	26%	34%	20%

Table 4

Effects of U247-51, U247-125, U247-73 and U247-90 on the gastric secretion of Shay rats. Intraperitoneal injection. Ligation period 5 hours. 5 rats to each dose.

Dose	Volume ml	pH	Gastric contents Titration values in meq	
			pH 3.5	pH 9.[illegible]
Control	7.4	1.1	0.647	0.758
U247-51 1.25 mg	1.9	2.13	0.320	0.630
U247-125 2.5 mg	8.3	1.27	0.583	0.686
U247-125 10 mg.	4.1	1.28	0.296	0.361
Control	7.8	1.07	0.558	0.64
U247-73 1.25 mg	2.6	1.80	0.128	0.175
U247-90 10 mg	4.3	1.34	0.312	0.383
U247-90 20 mg	2.3	1.82	0.125	0.173

the effect of metropin thus reducing the amount of acid secreted during the experimental period.

B. Non anticholinergic compounds

The effects of the two quaternary compounds U247-51 and U247-73 on Shay rats have been shown previously (ANTONSEN 1965a). They have an inhibitory effect on the acid secretion and the ulceration both when injected intraperitoneally and when administered into the gastro-intestinal canal. Comparisons with the corresponding tertiary compounds are shown in table 4. Ulceration cannot occur in so short a ligation period as 5 hours but the quaternary compounds have an effect 10 to 16 times greater on the acid secretion than the tertiary compound. The two quaternary compounds had approximately the same effect, but it seems as if U247-73 had a relatively stronger effect on the acid secretion than on the volume.

The effect of oral administration 3 hours before ligation are shown in table 5. It is seen that both the quaternary and the tertiary compounds had to be given in about 10 times larger doses in order to obtain the same effect as after intraperitoneal injection.

Statistical calculation, based on Student's test, of the difference between treated and untreated animals showed that of the doses having the same effect on the acid secretion whether administered orally or parenter

Table 5

Effects of U247-51, U247-125, U247-73, and U247-90 on the gastric secretion of Shay rats. Oral administration 3 hours before the ligation. Ligation period 5 hours. 5 rats to each dose.

Dose		Gastric contents: Volume ml	pH	Titration values in meq: pH 3.5	pH 9.2
Control		6.6	1.19	0.503	0.609
U247-73	5 mg.	6.1	1.84	0.202	0.307
U247-90	10 mg	7.3	1.16	0.530	0.694
U247-90	25 mg	9.8	1.20	0.645	0.790
Control		8.3	1.03	0.709	0.812
U247-73	5 mg	7.3	1.79	0.185	0.288
U247-90	50 mg	8.7	1.59	0.282	0.441
U247-51	10 mg	6.7	2.05	0.227	0.332
U247-125	100 mg	9.6	1.87	0.304	0.589

Table 6

Effects of U247-95, U247-96, and U247-73 given into the duodenum of Shay rats. In the 16-hour experiment 5 animals to each dose, and i the 8-hour experiment, 4 animals

Substance	Dose in mg	Period	Ulcer index	Gastric contents: Vol. ml	pH	Titration values in meq: pH 3.5	pH 9.2
Control		16 hrs.	3.0 × 100% = 300	12.8	1.54	0.637	0.891
U247-95	5	16 hrs.	1.6 × 80% = 128	11.5	1.74	0.599	0.832
U247-96	5	16 hrs.	0.5 × 40% = 20	10.9	1.59	0.444	0.663
U247-73	5	16 hrs.	1.2 × 40% = 48	11.2	1.44	0.589	0.814
Control		8 hrs.	1.0 × 75% = 75	11.7	1.37	0.773	0.998
U247-96	2.5	8 hrs.	0.25 × 25% = 6	8.4	1.30	0.616	0.772
U247-73	2.5	8 hrs.	0	7.5	1.63	0.534	0.726
U247-96	1.25	8 hrs.	0	5.8	1.42	0.426	0.571
U247-73	1.25						
U247-96	0.625	8 hrs.	0	7.7	1.47	0.598	0.736
U247-73	0.625						

ally the oral doses had considerably less effect on the volume than the doses intraperitoneally

C. Mixtures of anticholinergic and non anticholinergic compounds

Table 6 shows the effects obtained by administration of the compounds into the duodenum at the same time as ligation. Here too the quaternary compound U247–96 had a stronger effect than the corresponding tertiary compound (U247–95). U247–73 had approximately the same effect as U247–96, both on the secretion and ulceration. Simultaneous adminis-

Table 7

Test-meal experiment on rats. Comparison of U247–95 with U247–96 in cross-over test on 15 animals. Histamine 1.5 mg i.p. d is the difference between the mean values and t has been calculated by Student's t test.

		Control	U247 95 1 mg i.p.	U247 96 0.5 mg i.p.
HCl μeq.	$\bar{y}$	97.3	84.2	52.3
	d		13.1	45.0
	t		0.96	3.44
	p		0.1–0.5	<0.01
pH	$\bar{y}$	2.28	2.32	2.03
	d		0.04	0.33
	t		0.38	3.10
	p		>0.5	<0.01
V. L. secr. ml	$\bar{y}$	1.6	1.4	1.5
	d		0.2	0.1
	t		1.16	0.58
	p		>0.5	>0.5
V-p p. ml	$\bar{y}$	3.6	3.5	3.3
	d		0.1	0.3
	t		0.29	0.89
	p		>0.5	0.1–0.5
Phenol red per cent	$\bar{y}$	40	41	45
	d		1	5
	t		0.18	0.45
	p		>0.5	0.1–0.5

tration of half doses of the two compounds had a somewhat greater effect than a whole dose of each given separately while one-quarter doses of the two compounds combined had almost the same effect as the full doses given separately

In other words, an intensification of the effect is obtained by simultaneous administration of the two types of quaternary compounds.

Effects in test-meal experiments

A. Anticholinergic compounds

The effects of intraperitoneal doses are shown in table 7 The quaternary compound had a greater effect on the acid secretion than twice the dose of the tertiary compound. In the doses given none of the compounds had any effect on the volume or the emptying of the stomach.

The effects of 10 mg of the two compounds given by mouth, both 5 and 15 hours before the histamine-test meal, are shown in table 8 At no time did the tertiary compound have any significant effect, whereas the quaternary compound had an effect on the acid secretion both after 5 and 15 hours.

Further comparison of the quaternary with the tertiary compound is shown in table 9 1 mg atropine and 0.5 mg U247–96 had been given intraperitoneally 1 5 and 15 hours previously The given dose of atropine produced in the course of one hour a maximum dilation of the pupil and such a dry throat that it was difficult to pass a tube through it. The dryness had partly subsided after 5 hours, and after 15 hours the pupil was normal in size and there was no dryness left. An effect of atropine on the acid secretion was observed after one hour only U247–96 was found to have significant inhibitory effect on the acid secretion after 1 5 and 15 hours. Some dryness, much less than after atropine, was observed but only after one hour In other words, the actions of these two anticholinergic compounds differed very considerably

B Non anticholinergic compounds

As shown previously (ANTONSEN 1965 c), the two quaternary compounds have a marked inhibitory effect on the gastric secretion in test meal experiments, both after oral and after parenteral administration.

Table 10 shows the result of a direct comparison of U247–51 with the corresponding tertiary compound U247–125 To produce the same effect on the acid secretion the tertiary compound had to be administered

Table 8

Test meal experiments on rats. Comparison of U247–95 with U247–96 given by mouth 5 nd 15 hours previously Two cross-over tests on 15 animals. Histamine 1.5 mg i.p. d and t as in table 7

		5 hrs. before			15 hrs. before		
		Control	U247–95 10 mg orally	U247 96 10 mg orally	Control	U247–95 10 mg orally	U?47–96 10 mg orally
HCl µeq	$\bar{y}$	85.8	67 1	40.3	103.8	118.4	55..
	d		18.7	45.5		14.6	48 6
	t		1.28	3.35		0.64	..87
	p		0.1–0.5	<0.01		>0.5	<0 01
pH	$\bar{y}$	..41	2.57	2.93	..47	2.31	2.79
	d		0.16	0.52		0.16	0.32
	t		0.91	1 60		1.55	2.47
	p		>0.5	0.1–0.5		0.1–0.5	0 01–0 05
Vol secr ml	$\bar{y}$	1 4	1 5	1.2	1 6	1.3	1.3
	d		0.1	0.2		0.3	0.3
	t		0.52	1.05		0.77	0.81
	p		>0.5	0 1–0.5		0.1–0.5	0.1–0.5
V p p. ml	$\bar{y}$	3 7	3.6	3 1	4.3	4 0	3.9
	d		0.1	0.6		0.3	0.4
	t		0.30	1 76		0.60	0.93
	p		>0.5	0.1		>0.5	0.1–0 5
Phenol red per cent	$\bar{y}$	37	39	46	?9	32	34
	d			9		3	5
	t		0 33	1.53		0.53	0.91
	p		>0.5	0.1–0.5		0.1–0.5	0.1–0.5

doses about 16 times greater than the quaternary both intraperitoneally and orally When the compound was given orally 5 times larger doses than those given intraperitoneally were required to obtain the same effect. After intraperitoneal injection the quaternary compound brought about an augmentation of the volume secreted, whereas a slight reduction was seen in response to the tertiary compound. After oral administration the volume was slightly reduced in response to the quaternary compound and this considerably so in response to the tertiary compound. In all

Table 9

Test meal experiments on rats. Comparison of U247 96 with tropine given i.p. 1 5 and 15 hours previously Cross-over test on total of 15 imals. Histamine 1.5 mg i.p. d and t as in table 7

		1 hr before			5 hrs. before			15 hrs. before		
		Control	Atrop. 1 mg	U247 96 0 5 mg	C trol	Atrop. 1 mg	U247 96 0 5 mg	Control	Atrop. 1 mg	U 47 96 0.5 mg
HCl µeq	y	72.1	52.9	34 4	52.2	55 6	25.2	113 6	124 1	68 9
	d		19 2	37 7		1 4	27 0		10 7	44 7
	t		1.21	2.75		0 30	2.57		0.43	2.28
	p		0.1–0.5	0.01–0 05		0.5	0 01–0 05		>0.5	0.01–0.05
pH	y	2 72	2.87	3 06	2.72	2 67	3 56	2.54	2.50	2.81
	d		0.15	0.34		0.05	0 84		0.04	0.27
	t		0.50	1 13		0 26	2.08		0 28	1.80
	p		>0.5	0.1–0.5		>0.5	0 05–0.1		>0.5	0.09–0.1
Vol. secr ml	y	1 7	1.9	1.5	1.5	1.3	0.9	2 4	2.2	1 9
	d		0.2	0.2		0.2	0.6		0.2	0.5
	t		0 65	0.75		0.95	2.19		0.45	1.82
	p		>0.5	>0.5		0.1–0.5	0.01–0.05		>0.5	0.05–0.1
V p p. ml.	y	4.0	1 6	3.5	4.0	3.5	3.2	4 4	5 0	4 8
	d		0.4	0.5		0.5	0.8		0.6	0.4
	t		1 17	1 48		1 61	2.90		1 10	0.80
	p		0.1–0.5	0.1–0.5		0.1–0.5	0.01–0.05		0.1–0.5	0.1–0.5
Phenol red per cent	y	34	42	41	33	40	42	33	4	25
	d		8	7		7	9		9	8
	t		1.38	1 16		1.45	1.90		1 65	1.55
	p		0.1–0.5	0.1–0.5		0.1–0.5	0.05–0 1		0.1–0.5	0 1–0.5

Table 10

Test meal experiments on rats. Comparison of U247 51 with U247 1.5 gl en i.p. and by mouth. Two cross-over tests on 15 animals. Histamine 1.5 mg i.p.
d and t as I table 7

		Intraperitonally			Orally		
		Control	U247 51 1.25 mg	U247 125 20 mg	Control	U247 51 6.[illegible]5 mg	U247 1.5 100 mg
HCl μeq	$\bar{y}$	83 45	24 98	51.98	104 43	40.17	54 99
	d		58 47	31 47		64.[illegible]5	49 44
	t		4.03	2.11		5 74	4 39
	p		<0 01	0 01–0.05		<0 01	<0 01
pH	$\bar{y}$	2.62	4.25	[illegible]59	[illegible]18	3 46	2.76
	d		1 63	0.03		1.28	0.58
	t		3 17	0.11		3.20	2.5
	p		<0.01	0 1–0.5		<0.01	0.01–0 05
Vol. secr ml	$\bar{y}$	2.52	3 72	2.[illegible]1	2.83	2.36	1 90
	d		1.20	0.31		0.47	0 93
	t		2.47	0.74		1.04	[illegible]07
	p		0.01–0.05	0.1–0.5		0.1–0.5	0 01–0 05
V p.p. ml	$\bar{y}$	4.86	3 41	3.35	4 18	4.[illegible]6	2 84
	d		1 45	1.31		0.08	1.34
	t		3 39	4.58		0.16	[illegible]79
	p		<0.01	<0 01		0.5	<0 01
Phenol red per cent	$\bar{y}$	31.81	50.54	46 68	35 44	36.86	53.02
	d		18.73	14.87		1 4[illegible]	17.58
	t		2.69	2.55		0.36	3 01
	p		0 01–0.05	0.01–0 05		>0.5	<0 01

cases the tertiary compound greatly restricted the gastric emptying, whereas the quaternary compound had no such effect when administered by mouth

Discussion

All the compounds tested had an inhibitory effect on the gastric secretion of rats. The quaternary compounds always had a stronger effect than the corresponding tertiary compound The investigations showed however that a direct quantitative comparison was difficult

The individual compounds differ greatly both in their absorption and in their effect on the acid secretion, secretion of gastric juice, and gastric emptying.

It is well-known that the quaternary compounds are less readily absorbed from the gastro-intestinal canal than the tertiary compound (LEVINE *et al* 1955 LEVINE & CLARK 1957) The relation between the effects of quaternary and tertiary anticholinergic compounds in animal experiments depends on the test used (LUDUENA & LANDS 1954 URSILLO & CLARK 1955). In the above-mentioned experiments on Shay rats the quaternary compounds were absorbed in sufficient amounts to demonstrate that, also when administered into the gastro-intestinal canal, larger amounts are required of the tertiary than of the quaternary compound to obtain the same effect. The cause of this is possibly the more prolonged action of the quaternary compound, the duration of the action having been demonstrated as being of great importance. To ensure in such experiments that one is not examining local actions, the compounds used in the Shay test must be given a few hours before ligation, a procedure which also requires prolonged action of the compounds. Atropine was found to have a smaller effect on the stomach than might have been expected considering its prolonged and marked mydriatic action.

Simultaneous administration of quaternary anticholinergic and non-anticholinergic compounds into the duodenum had a greater effect than was to be expected from the effects of the two types given separately The non-anticholinergic compounds were prepared in our chemical laboratory since we hoped that by simultaneous oral administration of these compounds we might be able to improve the absorption of active quaternary compounds. An increased effect of metropin ® has been shown previously (ANTONSEN 1965a) Contrary to expectation the compounds were found to have independent inhibitory effects on the stomach. CAVALITO & O DELL (1958) have shown however that one can increase the absorption of active quaternary compounds by means of the inactive quaternary type

The test meal method, which is much better for studying the effect of orally administered compounds, has also shown a stronger action of the quaternary anticholinergic compound than of the tertiary compound The difference cannot be due to a difference in duration of action since the experimental period was only 45 minutes, and the experiments also demonstrated that administration at different times previously did not alter the conditions observed. The effect on the gastric secretion was not parallel to the peripheral anticholinergic action.

In the test-meal experiments with the non-anticholinergic compounds, doses were compared with approximately identical inhibitory effects on

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Comparison of the Stimulating Effects of Gastrin and Histamine on the Gastric Secretion of Rats

By

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A stimulating effect of gastrin on the gastric secretion has been demonstrated in many different ways in experiments on dogs, cats, rats, and man.

GHOSH & SCHILD's (1958) method of measuring the secretion in anaesthetised animals has most frequently been used for standardisation on rats. Rats with chronic gastric fistula (ADASHEK & GROSSMAN 1963 SCHOENFIELD *et al* 1966) and rats with ligated stomach (SMITH 1966) have been used to demonstrate the effect on non-anaesthetised animals. KAHLSON *et al* (1964) have demonstrated an effect by means of THORNTON & CLIFTON's (1959) test-meal method Previous experiments with histamine (ANTONSEN 1965b) showed this method to be particularly useful. It has therefore been used for a further investigation on the effect of gastrin in rats.

In addition, rats with ligated stomach have been used (Shay rats). In previous experiments we found the acid secretion of these rats to be so strong as to render the animals unsuitable for studying a possible stimulating effect (ANTONSEN 1965a). BAUME & LAW (1965) observed however that during the first two hours of the ligation period histamine has a stimulating effect, which changes to an inhibitory effect during the next two hours. Further KIM & SHORE (1963) have shown that by simultaneous ligation of the oesophagus, the secretion is depressed so much that the animals concerned can be used for demonstrating the effects of stimulating drugs. While comparing gastrin with histamine we have taken the opportunity of studying these problems.

Methods

The *test-meal method* was used in the same manner as described previously (ANTONSEN 1965b).

Sherman rats were used as experimental animals, some as in previous experiments (ANTONSEN 1953, ANTONSEN & NIELSEN 1963) and others with additional ligation of the oesophagus. After an abdominal incision a ligature was put round the pyloric sphincter. Then, after an incision into the neck, the oesophagus was ligated on a level with the larynx. Here care should be taken not to injure the vagus nerves. After both incisional wounds had been sutured, the animals were returned to their wire cages. All the doses were given intraperitoneally immediately after ligation.

Drugs

Histamine was administered as the hydrochloride dissolved in 0.9 % NaCl. The gastrin preparation used was a highly purified preparation produced at Leo's [illegible] Copenhagen. It was standardized biologically according to LAI's (1964) method [illegible] communication by O. BEHRENS). Batch no. 65031 containing 160 arbitrary units per ampoule was used throughout the experiments.

Results

A Test-meal experiments

The average results of a series of experiments are shown in Table 1. The amount of acid secreted increased proportionally with the dose of

Table 1

Effects of gastrin and histamine in test-meal experiments. All the doses were given [illegible] together with the test meal.

N is the number of rats. Phenol red per cent is the percentage of the test meal recovered at the end of the experimental period. V p.p. is the amount passing through the pyloric sphincter during the experimental period (45 min.). These two figures [illegible] gastric emptying.

Dose	N	HCl μeq.	pH	Phenol red per cent	V p.p. ml
Control	2[illegible]	53.4	2.95	[illegible]	4.c
Histamine 0.75 mg	12	118.2	2.25	41.4	3.44
Histamine 1.5 mg	21	127.6	2.25	41.5	3.43
Histamine 3.0 mg	12	1[illegible]4.5	2.18	[illegible]	3.56
Gastrin 0.5 u.	12	[illegible].1	2.59	[illegible]	4.20
Gastrin 1.0 u.	24	102.3	2.52	[illegible]	4.25
Gastrin 2.0 u.	20	117.2	2.38	[illegible]	4.25
Gastrin 4.0 u.	16	142.6	2.24	[illegible]	3.77
Gastrin [illegible].0 u.	14	179.3	2.12	[illegible]	4.00

Table 2

Cross-over test between histamine and gastrin in test meal experiments. 12 animals to each dose.

The doses were given i.p. immediately after the test meal. Phenol red and V.p.p. as in table 1 d is the difference between the mean values, and t has been calculated by Student's test.

		Control	Histamine 3 mg	Gastrin 4 u.	Gastrin 8 u.
HCl μeq	$\bar{y}$	41.0	124.5	126.2	169.5
	d		83.5	85.2	128.5
	t		6.2	5 1	13.4
	p		<0.01	<0.01	<0.01
pH	$\bar{y}$	3 18	2.18	2.33	2.21
	d		1 00	0.85	0.97
	t		4.8	3 7	4.1
	p		<0.01	<0.01	<0.01
Vol. secr ml	$\bar{y}$	2.08	2.43	2.33	2.36
	d		0.35	0.25	0.28
	t		1 6	0.9	0.8
	p		0.1–0.5	0.1–0.5	0.1–0 5
V p.p. ml	$\bar{y}$	4.02	2.94	3.78	4 07
	d		1.08	0.24	0 05
	t		3 7	0 7	0.1
	p		<0.01	0.1–0.5	~0.5
Phenol red per cent	$\bar{y}$	35 9	53.3	39 4	35.1
	d		17 4	3.5	0 8
	t		4.[illegible]	0 9	0.7
	p		<0.01	0 1–0.5	~0.5

gastrin, and the maximum effect of this drug greatly exceeded that of histamine. As in previous experiments (ANTONSEN 1965b) the acid secretion was no greater with 3 mg than with 1.5 mg histamine The histamine doses used produced a marked delay in gastric emptying. This was not seen even after the largest dose of gastrin.

The straight line representing the relation between the logarithm of the dose of gastrin and the acid secretion has the following formula

$$y = 74\,7X + 102.3$$

Table 3

Effects of gastrin and histamine on Shay rats in 2-hour and 4-hour experiments.

Operation V comprises the experiments with ligation at the pyloric sphincter only. In the experiments marked V + O the oesophagus was also ligated.

Operation	Dose	Duration in hrs.	N	Volume ml	pH	Titration values in meq	
						pH 3.5	pH 9.2
	Control	2	28	3.49	2.25	0.161	[illegible]
	Histamine 1.5 mg	2	13	2.31	2.31	0.078	0.143
V	Histamine 2.0 mg	2	11	2.32	2.15	0.085	0.155
	Histamine 3.0 mg	2	9	2.37	2.24	0.117	0.182
	Gastrin 4 u.	2	8	4.41	1.75	0.303	0.383
	Control	2	14	0.54	2.30	0.033	0.053
V + O	Histamine 1.5 mg	2	6	1.00	1.70	0.068	0.093
	Histamine 2.0 mg	2	8	1.25	1.77	0.088	0.119
	Gastrin 4	2	8	0.86	2.08	0.080	0.106
	Control	4	8	5.40	1.62	0.468	0.581
V	Histamine 1.5 mg	4	8	5.55	1.56	0.510	0.634
	Gastrin 4 u.	4	7	7.45	1.50	0.652	0.800
	Control	4	8	0.46	2.39	0.023	0.042
V + O	Histamine 1.5 mg	4	8	1.04	1.96	0.079	0.101
	Gastrin 4 u.	4	8	1.04	2.12	0.045	0.069

Table 2 shows the average results from a cross-over test between gastrin and histamine. 4 u. gastrin had approximately the same effect on the acid secretion as 3 mg histamine. While histamine was seen to cause a highly significant delay of gastric emptying, this was not seen either after 4 or after 8 u. gastrin. None of the doses given caused any significant alteration in the volume secreted.

B. *Shay rats*

The results of the experiments on Shay rats are shown in table 3.

Ligation of the stomach both at the oesophagus and at the pyloric sphincter gave a much lower secretion than ligation at the pyloric

Table 2

Cross-over test between histamine and gastrin in test-meal experiments. 12 animals in each dose.

The doses were given i.p. immediately after the test meal. Phenol red and V p.p. as in table 1. d is the difference between the mean values and t has been calculated by Student's test.

		Control	Histamine 3 mg	Gastrin 4 u.	Gastrin 8 u.
HCl µeq	ȳ	41.0	124.5	126.2	169.5
	d		83.5	85.[illegible]	128.5
	t		6.[illegible]	5.1	11.4
	p		<0.01	<0.01	<0.01
pH	ȳ	3.18	2.18	2.33	2.21
	d		1.00	0.85	0.97
	t		4.8	3.7	4.1
	p		<0.01	<0.01	<0.01
Vol. secr. ml	ȳ	[illegible].08	2.43	2.33	2.36
	d		0.35	0.25	0.28
	t		1.6	0.9	0.8
	p		0.1–0.5	0.1–0.5	0.1–0.5
V p.p. ml	ȳ	4.02	2.94	3.78	4.07
	d		1.08	0.24	0.05
	t		3.7	0.7	0.1
	p		<0.01	0.1–0.5	>0.5
Phenol red per cent	ȳ	35.9	53.3	39.4	35.1
	d		17.4	3.5	0.8
	t		4.2	0.9	0.7
	p		<0.01	0.1–0.5	>0.5

gastrin and the maximum effect of this drug greatly exceeded that of histamine. As in previous experiments (Antonsen 1965b), the acid secretion was no greater with 3 mg than with 1.5 mg histamine. The histamine doses used produced a marked delay in gastric emptying. This was not seen even after the largest dose of gastrin.

The straight line representing the relation between the logarithm of the dose of gastrin and the acid secretion has the following formula

$$y = 74.7X + 102.3$$

Table 3

Effects of gastrin and histamine on Shay rats in 2-hour and 4-hour experiments.

Operation V comprises the experiments with ligation at the pyloric sphincter only. In the experiments marked V + O the oesophagus was also ligated.

Operation	Dose	Duration in hrs.	N	Volume ml	pH	Titration values in meq pH 3.5	Titration values in meq pH 9.2
V	Control	2	28	3.49	2.25	0.161	0.252
	Histamine 1.5 mg	2	13	2.31	2.31	0.078	0.143
	Histamine 2.0 mg	2	11	2.32	2.15	0.085	0.155
	Histamine 3.0 mg	2	9	2.37	2.24	0.117	0.182
	Gastrin 4 u.	2	8	4.41	1.75	0.303	0.383
V + O	Control	2	14	0.54	2.30	0.033	0.053
	Histamine 1.5 mg	2	6	1.00	1.70	0.068	0.093
	Histamine 2.0 mg	2	8	1.25	1.77	0.088	0.119
	Gastrin 4 u.	2	8	0.86	2.08	0.090	0.106
V	Control	4	8	5.40	1.62	0.468	0.581
	Histamine 1.5 mg	4	8	5.55	1.56	0.510	0.634
	Gastrin 4 u.	4	7	7.45	1.50	0.652	0.800
V + O	Control	4	8	0.46	2.39	0.023	0.042
	Histamine 1.5 mg	4	8	1.04	1.96	0.079	0.101
	Gastrin 4 u.	4	8	1.04	2.12	0.045	0.069

Table 2 shows the average results from a cross-over test between gastrin and histamine. 4 u. gastrin had approximately the same effect on the acid secretion as 3 mg histamine. While histamine was seen to cause a highly significant delay of gastric emptying, this was not seen either after 4 or after 8 u. gastrin. None of the doses given caused any significant alteration in the volume secreted.

B. *Shay rats*

The results of the experiments on Shay rats are shown in table 3.

Ligation of the stomach both at the oesophagus and at the pyloric sphincter gave a much lower secretion than ligation at the pyloric

sphincter only In agreement with previous observations histamine produced no stimulation on the normal Shay rats, but there was some increase of the very low secretion in the animals in which the oesophagus had been ligated. Gastrin, on the other hand, stimulated the secretion in both groups of Shay rats. In the present experiments histamine did not have a more marked effect in the 2-hour than in the 4-hour experiments. This is contrary to BAUME & LAW s (1965) findings.

Discussion

The experiments showed that a stimulating effect can be demonstrated by gastrin and histamine on the gastric secretion of non-anaesthetised rats, no matter whether the Shay test or the test-meal method is used.

The test meal method is best suited for measuring the activity of the gastrin preparation. Linearity is seen here between the dose and the effect on the acid secretion. UVNAS & EMÅS (1961), in experiments on non-anaesthetised cats, found parallel dose response curves for gastrin and histamine thus allowing the gastrin preparation to be standardised, with histamine as standard. In the present experiments no linearity was found between the dose and the effect of histamine on rats. BARRET *et al* (1966) observed that the method of anaesthesia has a considerable influence on the effect of gastrin on anaesthetised rats. ADASHEK & GROSSMAN (1963), in experiments on rats with chronic fistula, similarly found linearity between the dose and the effect of gastrin, and, further a much greater maximum effect of gastrin than of histamine SHOENFIELD *et al* (1966), on the other hand, found no linearity in the cases of rats with fistula given an impure gastrin preparation.

With the Leo Gastrin preparation used in the present experiments CHRISTIANSEN (1966) in clinical studies, obtained results which differed from those with pure gastrin BARABAS *et al* (1966), in clinical experiments with Leo Gastrin obtained the same secretion curves as with histamine.

On comparing the effects of gastrin and histamine by aspiration of gastric secretion it is important to realise that histamine causes a delay of the gastric secretion, which is not noticed after gastrin. This difference is not demonstrable in experiments on rats with fistula, on anaesthetised rats according to GHOSH & SCHILD s method (1955) or on Shay rats. If the same difference between the effects of the two drugs can be demonstrated in the human stomach, this should be important in assessing clinical results.

The results of experiments on Shay rats confirmed that ligation of the oesophagus brings about an excessive reduction of the secretion According to LEVINE (1965) this is solely due to the abolition of the stimulating effect of saliva. BRODIE & KNAPP (1965) on the other hand, believe that it is the accumulated saliva which induces a vagal inhibitory action.

Though stimulating effects of histamine and gastrin can be demonstrated in rats with ligated oesophagus and stomach, the method is not particularly suitable for this purpose. The basal secretion is very low and measurement of this subject to considerable errors. The volume secreted is not augmented by prolonging the ligation period from 2 to 4 hours, and a further prolongation is probably harmful. It was clear that the animals exposed to double ligation were far more stressed than those with a single ligation. Further a great disadvantage of this method is that, unlike the other methods, it does not allow a comparison between the effects of different doses on the same animal.

Summary

The stimulating effects of histamine and of a gastrin preparation on the gastric secretion of rats have been compared.

Using a test-meal method, proportionality was seen between the dose of gastrin and the degree of acid secretion. This was not observed with the histamine doses given In cross-over tests 4 u. gastrin and 3 mg histamine were found to have approximately the same effect on the gastric secretion but gastrin did not bring about such a delay of the gastric emptying as histamine.

It was shown in experiments on Shay rats that simultaneous ligation of the pyloric sphincter and the oesophagus caused a considerable reduction of the acid secretion. Histamine was found to increase the secretion in the animals with double ligation. Gastrin had a stimulating effect in both the single and the double-ligated animals. Prolongation of the experimental period from 2 to 4 hours did not augment the gastric secretion of the animals with ligated oesophagus. Further histamine did not have a more marked effect in the 2 hour experiments than in those lasting 4 hours.

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Antagonism of Gastrin- and Histamine-Stimulated Gastric Secretion in Rats

By

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A test-meal method has been used for the quantitative determination of the stimulating effects of both histamine and gastrin on the gastric secretion in rats (ANTONSEN 1967 b)

Using this method a difference was observed between the dose response curves of these two drugs, and histamine was shown to cause a delay in the gastric emptying not seen after gastrin.

Previous investigations (ANTONSEN 1965 b) showed that the antihistamine antazoline may bring about an increase in the histamine-stimulated secretion. Anticholinergic substances and some quaternary ammonium compounds, on the other hand, were found to have a marked antagonistic effect (ANTONSEN 1965 c).

Comparisons have been made of the effects on the gastrin-stimulated secretion in order to find out whether the actions of these antagonists are similar when stimuli other than histamine are used.

Methods

The test-meal method was used in the same way as previously described (ANTONSEN 1965 b).

Drugs

Histamine was used in the form of the hydrochloride. The gastrin used was a preparation from Løvens Kemiske Fabrik, batch 65031 which contains 160 units per ampoule (ANTONSEN 1967 b). Atropine was used in the form of the sulphate, and antazoline as the preparation antistina ® diluted with 0.9% NaCl. In addition, the quaternary anticholinergic drug mestropin ® and two quaternary non-anticholinergic compounds, U247–51 and U247 73, were used, as described previously (ANTONSEN 1965   1967).

Table 1

Effects of antazoline and atropine on gastrin-stimulated acid secretion in test-meal experiments on rats. Antazoline and atropine given subcutaneously two hours before the test meal. Gastrin, 2 u., given intraperitoneally immediately after the test meal. Cross-over test with 12 animals for each dose. Phenol red per cent is the percentage of the test meal recovered in the stomach at the end of the experimental period. V p.p. is the amount of gastric juice passing through the pyloric sphincter during the experimental period (45 min.). d is the difference between the mean values, and t has been calculated by Student's t-test.

		Control	Antazoline 5 mg subc.	Atropine 5 mg subc.
HCl μeq.	$\bar{y}$	132.7	113.5	33.4
	d		19.2	99.3
	t		1.19	10.95
	p		0.1–0.5	<0.01
pH	$\bar{y}$	2.26	2.24	3.49
	d		0.02	1.23
	t		0.06	3.41
	p		>0.05	<0.01
Vol. secr. ml	$\bar{y}$	2.28	2.82	2.48
	d		0.54	0.20
	t		1.27	0.36
	p		0.10–0.5	>0.5
V p.p. ml	$\bar{y}$	4.15	4.12	4.45
	d		0.03	0.30
	t		0.08	0.514
	p		>0.5	>0.5
Phenol red per cent	$\bar{y}$	35.4	37.6	36.1
	d		2.[illegible]	0.07
	t		0.5	0.2
	p		>0.5	>0.5

Results

Table 1 shows that atropine had a marked antagonistic effect on the gastrin-stimulated secretion of acid, whereas the same dose of antazoline had no such effect. None of the drugs had any effect on the amount of juice secreted or on the rate of gastric emptying.

In table 2 the effects of antazoline on gastrin- and histamine-stimulated

Table 2

Comparison of the effects of antazoline on histamine- and gastrin-stimulated gastric secretion in test-meal experiments.

Eight animals to each dose. Antazoline given subcutaneously $2\frac{1}{2}$ hours before the test-meal Histamine, 1.5 mg. gastrin, 2 u., given intraperitoneally immediately after the test-meal. Phenol red, V p.p., d, and t as in table 1

		Gastrin stimul.		Histamine stimul.	
		Control	Antazoline 5 mg	Control	Antazoline 5 mg
HCl μeq	ȳ	93.9	97.6	118.5	134.5
	d		5.7		16.0
	t		0.15		1.3
	p		>0.5		0.1–0.5
pH	ȳ	2.57	2.62	40	2.38
	d		0.05		0.02
	t		0.01		0.24
	p		>0.5		>0.5
Vol. secr. ml	ȳ	2.81	3.11	2.59	2.84
	d		0.30		0.25
	t		0.56		0.55
	p		>0.5		>0.5
V p.p.	ȳ	4.40	4.86	3.53	4.33
	d		0.46		0.80
	t		0.98		1.37
	p		0.1–0.5		0.1–0.5
Phenol red per cent	ȳ	34.1	30.5	45.0	36.6
	d		3.6		8.4
	t		0.95		1.33
	p		0.1–0.5		0.1–0.5

secretion respectively have been compared. As had been observed previously (ANTONSEN 1965b), antazoline caused some increase in the acid secretion in the histamine-treated rats. This was not found in the gastrin-treated animals. Among the control animals, the gastric emptying was slower in the histamine-treated than in the gastrin-treated, but the effect of antazoline on the rate of gastric emptying was the same in the two experimental series.

Table 3

Effects of metropin, U247 51 a d U247 73 on gastrin-stimulated gastric secretion in test meal experiments.

The test compounds given intraperitoneally three hours before, and gastrin, 4 µ., given intraperitoneally immediately fter the test-meal.
Eight animals to each dose. Phenol red, V p.p., d, and t as in table 1

		Control	Metropin 5 mg	U247–73 1 mg	U247–51 1 mg
HCl µeq	y	119 4	19.0	15.5	7.8
	d		100.4	103.9	111.6
	t		3 93	3.83	3.07
	p		<0.01	<0.01	0.01–0 05
pH	y	..34	4.07	4.70	6 00
	d		1 73	..36	3.66
	t		6.09	5.57	12.78
	p		<0.01	<0 01	<0.01
Vol. secr ml	y	2.10	1 67	..48	2.73
	d		0.43	0.38	0.63
	t		0.96	1 10	1 41
	p		0.1–0.5	0.1–0.5	0.1–0.5
V p.p. ml	y	3 97	3 18	4.62	4.31
	d		0.79	0.65	0 34
	t		1.60	1.53	0 56
	p		0.1–0.5	0.1–0.5	>0.5
Phenol red per cent	y	33 6	45 6	29.9	28.0
	d		12.0	3.7	4 4
	t		1 87	0.55	0.50
	p		0.05–0.1	>0.5	>0.5

In table 3 it is seen that U247–51 and U247–73 as well as metropin ® have a marked inhibitory effect on the acid secretion when given intraperitoneally three hours before gastrin. Neither U247–51 nor U247–73 caused any delay in the gastric emptying, and there was only a slight increase in the volume secreted. In experiments with histamine-stimulated gastric secretion we have found previously that these antagonists have a maximum effect on the acid secretion when given three hours before the stimulation, while the delaying effect on the gastric emptying and the in-

Table 4

Comparison of the effects of U247 51 and U'47 73 on histamine and gastrin-stim lated gastric secretion in test meal experiments. The test compounds given intraperitoneally one hour before. Gastrin, 4 u., and histamine, 3 mg. given intraperitoneally immediately after th test meal. Six animals for each dose.

C represents mean values for control animals, and T for animals treated with antagonist. Phenol red, V p p., d and t as in table 1

	Antagonist		C	T	d	t	p
Histamine stimul.	U247 51 0.5 mg	HCl μeq	15_7	121.0	31 7	1 06	0.1–0.5
		pH	2.21	2.46	0.25	1.92	0.05–0.1
		Vol. secr ml	3.19	4 37	1 18	0 80	0.1–0.5
		V p.p. ml	4.57	3 83	0.74	1.20	0.1–0.5
		Phenol red per cent.	33	52	19	2.75	0.01–0.05
	U247 73 0.5 mg	HCl μeq.	152.7	91 5	61.2	2.19	0.05–0.1
		pH	2.21	2.53	0.32	2.70	0.01–0 05
		Vol. secr ml	3 19	2.53	0 66	1 12	0.1–0.5
		V p p. ml	4.57	3.34	1.23	2.17	0 05–0.1
		Phenol red per cent	33	48	15	2.24	0.01–0.05
Gastrin stimul.	U247 51 0.5 mg	HCl μeq	271.9	36.7	235.2	7.33	<0 01
		pH	1.90	3.87	1 97	3.20	0.01–0.05
		Vol. secr ml	2.74	1.92	0.82	1 17	0.1–0.5
		V p.p. ml	4.95	3.11	1.84	6.03	<0.01
		Phenol red per cent.	29	45	16	1.95	0.05–0.1
	U 47–73 0.5 mg	HCl μeq	271 9	36.1	235.7	8.01	<0.01
		pH	1.90	3.73	1.83	4.98	<0.01
		Vol. secr ml	2.74	3.06	0.32	0.36	>0.5
		V.p.p. ml.	4 95	4.57	0.38	0.95	0.1–0.5
		Phenol red per cent.	29	30	1	0.20	>0.5

crease in the volume secreted was seen after one hour and had disappeared by 3 hours (ANTONSEN 1965c) This is also seen to be the case with the gastrin-stimulated secretion While in this experiment U247–51 showed a more marked inhibitory action than U247–73 the reverse was obtained in all the experiments with histamine.

A direct comparison of the effects of the two quaternary non-anticholinergic drugs on histamine- and gastrin-stimulated secretion is shown in table 4 The doses used were only half of those given in the previous experiment, and the compounds were administered one hour instead of three before the test.

The dose of gastrin (4 u.) given to the controls increased the acid secretion more than did the histamine dose (3 mg) Nevertheless the two compounds were more antagonistic to gastrin than to histamine. As usually U247–73 had a more marked antagonistic effect than U247–51 on histamine, whereas the two compounds were about equally effective against gastrin

In the experiments with histamine the two compounds caused a certain delay in the gastric emptying, while in the gastrin experiments U247–51 had a marked delaying effect while U247–73 had no such effect.

Discussion

In test-meal experiments on rats anticholinergic compounds were found to have a marked antagonistic effect on the gastrin-stimulated gastric secretion, similar to that found previously on histamine stimulated secretion.

Such a pure antihistamine as antazoline antagonizes neither histamine- nor gastrin-stimulated secretion. The increase in the acid secretion seen when the antihistamine is given before histamine is not observed when gastrin is given as stimulant It has been shown previously that histamine causes a delay in gastric emptying which is not seen after gastrin (ANTONSEN 1967b). In the present experiments it appears as though the antihistamine prevented this effect. This is possibly the explanation of the difference between the effects of an antihistamine on histamine- and gastrin-stimulated secretion.

The quaternary non-anticholinergic compounds have a stronger antagonistic effect on the gastric secretion stimulated by gastrin than on that stimulated by histamine The cause of this is unknown. Most investigators believe, however that gastrin acts on the gastric secretion *via* a liberation of endogenous histamine (HAVERBACK *et al* 1965), a process which presumably facilitates a blocking of the action.

The inhibitory action of these drugs on the gastric secretion doubtless involves a certain sympathomimetic effect (ANTONSEN 1965c) HARRIES (1956 & 1957) has shown that nor-adrenaline inhibits the gastric secretion to an extent dependent on the kind of stimulation used

The sympathomimetic effect is characterised by an increase in the non parietal gastric secretion and in the bile production (THOMPSON & VANE 1953 HARRIES 1956) When U247–51 which has a much stronger sympathomimetic effect than U247–73 is given three hours before the experiment, 5–6 ml of bile-coloured gastric juice can be aspirated from the stomach as compared with 1–2 ml of clear juice found normally

If as the results of the experiments suggest, sympathomimetic drugs are characterised by being highly antagonistic to gastrin-stimulated secretion, this also explains why U247–51 has a relatively stronger action against gastrin than against histamine.

Summary

Using a test-meal method on rats, we have studied the influence of pretreatment with anticholinergic drugs, antihistamines, and some quaternary non-anticholinergic compounds on histamine and gastrin-stimulated gastric secretion.

Both atropine and the quaternary anticholinergic compound metropin ® had a marked antagonistic effect on the gastrin-stimulated secretion. The antihistamine antazoline had no effect on the gastrin-stimulated secretion No increase was observed in the acid secretion similar to that seen in relation to histamine stimulated secretion.

Two quaternary non-anticholinergic compounds, U247–51 and U247–73 which have a marked antagonistic effect on histamine-stimulated secretion, were found to be even more antagonistic to gastrin. While U247–73 always has a more marked antagonistic effect than U247–51 on histamine-stimulated secretion U247–51 which has a considerable sympathomimetic action, has the same antagonistic effect as U247–73 to gastrin-stimulated secretion

The influence of the test compounds used on the rate of gastric emptying doubtless bears some relation to the effect observed on the gastric secretion.

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Subchronic Toxicity of some Erythromycin Compounds in Rats

By

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Clinical experiments have shown that erythromycin has a low toxicity (HAIGHT & FINLAND 1952 HEILMAN *et al.* 1952 SHOEMAKER & YOW 1954 SOLOMON & JOHNSTON 1955)

Investigations in experimental animals have confirmed this view (MCGUIRE *et al* 1952), with the exception however of guinea pigs and hamsters which die within a short time of administration (KAIPAINEN & FAINE 1954 TIOERTT & GOCHENOUR 1957).

Erythromycin base decomposes readily under the influence of hydrochloric acid (SMITH *et al* 1953 KIRBY *et al* 1953 JOSSELYN & SYLVESTER 1953), but propionyl erythromycin lauryl sulphate on oral administration, gives higher serum levels of the antibiotic than the erythromycin base and some of its other compounds (KUDER 1960 GRIFFITH 1960 GRIFFITH & BLACK 1962). At first it seemed that there were hardly any side effects with propionyl erythromycin lauryl sulphate (KUDER 1960), but soon there were reports of patients with hepatitis of the cholestatic type following its administration (KOEHLSTAEDT 1961 JOHNSON & WENDELL 1961 ROBINSON 1961 HAVENS 1962)

This type of hepatitis has not been noted, clinically at least, in connection with the use of other erythromycin compounds on the other hand there is no satisfactory explanation to account for the hepatotoxic effect of propionyl erythromycin lauryl sulphate

The present study compares the effects produced by erythromycin base (EB) erythromycin lactobionate (EL) and propionyl erythromycin lauryl sulphate (PELS) in growing rats, and the serum levels of the antibic obtained with these compounds on oral administration.

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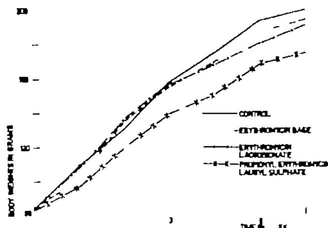


Fig. 1 The weekly increase in mean weight of rats treated with propionyl erythromycin lauryl sulphate, erythromycin base and erythromycin lactobionate for six weeks. The daily oral dose was equivalent to 800 mg erythromycin/kg body weight.

Serum enzymes

The alkaline phosphatase and glutamic pyruvic transaminase of the serum in the different groups at the end of the experiment are shown diagrammatically in figs. 2 and 3

The alkaline phosphatase of the serum in the EB group at the end of

Table 1

Mean weights of animals and livers in different groups treated with propionyl erythromycin lauryl sulphate, erythromycin base and erythromycin lactobionate for six weeks. The daily oral dose corresponded t 800 mg erythromycin/kg body weight. The mortality during treatment is also shown.

Treatment	Weights of animals (g) Mean ± SEM		Weights of livers (g)	No. of survivals
	Before treatment	After six weeks	Mean ±SEM	
Control	84 ± 1.5	200 ± 4.4	8.0 ± 0.3	25/30
Propionyl erythromycin lauryl sulphate.	83 ± 1.9	176 ± 5.8	9.2 ± 0.3	13/20
Erythromycin base	85 ± 1.9	195 ± 4.5	7.6 ± 0.3	10/20
Erythromycin lactobionate	84 ± 1.4	192 ± 8.0	8.3 ± 0.3	13/20

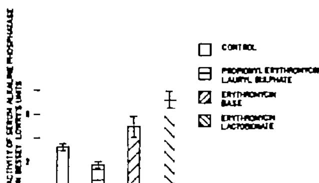


Fig. 2. Mean serum alkaline phosphatase and SEM i different animal groups treated for six weeks with propionyl erythromycin lauryl sulphate, erythromycin base and erythromycin lactobionate corresponding to 800 mg erythromycin/kg body weight.

the experiment was 5 0 Bessey Lowry units (SEM 0.9), and the glutamic pyruvic transaminase of the serum was 236 Wroblewski's units (SEM 7).

For the rats treated with EL, the corresponding values were 7.3 Bessey-Lowry units (SEM 0 7) and 308 Wroblewski's units (SEM 6).

For the animals of the PELS group, the mean alkaline phosphatase of the serum was 1 7 Bessey-Lowry units (SEM 0.3) and the glutamic pyruvic transaminase was 300 Wroblewski's units (SEM 11)

The values for the control group were 3.3 Bessey Lowry units (SEM 0.3) and 260 Wroblewski's units (SEM 6) respectively

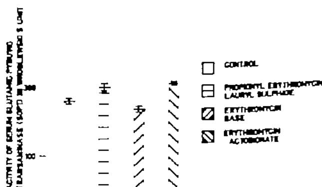


Fig. 3 Mean serum glutamic pyruvic transaminase (SGPT) and SEM in different animal groups treated fo six weeks with propionyl erythromycin lauryl sulphate, erythromycin base and erythromycin lactobionate. The daily oral dose was equivalent to 800 mg er mycin/kg body weight.



Fig. 4 Skin of rat treated with propionyl erythromycin lauryl sulphate for six weeks. The daily oral dose was equivalent t 800 mg erythromycin/kg body weight. Haematoxylin-Eosin × 100.

Histological findings

Haematoxylin-eosin staining revealed no significant changes in the liver kidneys and adrenals of the test animals of the various groups as compared with the control group

In the skin sections of the rats of the PELS group the hair follicles were markedly atrophied and the epidermis was hyperkeratotic. Slight para keratosis was also visible (fig. 4).

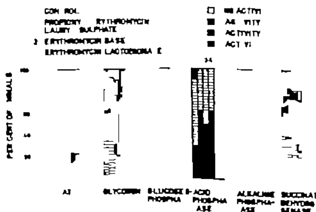


Fig. 5 Graphic presentation of the content of fat nd glycogen and enzyme activities in livers of different groups treated for six weeks with propionyl erythromycin lauryl sulphate, erythromycin base and erythromyci lact bionate. The daily oral dose was equivalent to 800 mg erythromycin/kg body weight.

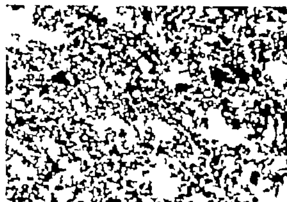


Fig. 6. Liver of rat treated with erythromycin base f six weeks. Fatty degeneration. Oil Red-O staining × 400.

Histochemical findings

The fat and glycogen present in the liver and the staining activity of the enzymes studied are shown in fig. 5 Staining for fat revealed that the animals of the EB group showed a fairly marked increase in fat compared with the other groups. The fat was present in the form of tiny drops in the cellular plasma and the intercellular spaces. The changes were local over relatively small areas (fig. 6) Fig. 5 shows, further that differences in the amount of glycogen between the animals of the different groups were negligible.

The activity of glucose-6-phosphatase was highest in the animals of the



Fig. 7 Liver of rat treated daily for six weeks with propionyl erythromycin lauryl sulphate. Alkaline phosphatase activity in biliary capillaries, canaliculi and ducts. Frozen section fixed in Formol-$CaCl_2$-Macrodex for 10 min., incubation time 30 min. × 400.

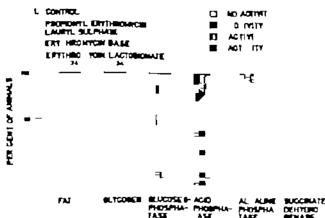


Fig. 8. Graphic presentation of the content of f t and glycogen and enzyme activities in kidneys of different groups treated for si weeks with propionyl erythromycin lauryl sulphate, erythromycin base and erythromycin lactobionate. The daily oral dose was equivalent t 800 mg erythromycin/kg body weight.

PELS group while there was none in the liver of the EB group (fig. 5) Acid phosphatase was slightly increased in all experimental groups as compared with the control group. There was hardly any activity of alkaline phosphatase in the bile ducts of the control animals, while 20–30 / of the animals of the PELS group and the EL group showed some activity This occurred in the biliary capillaries, canaliculi and ducts, while the hepatic cells were almost free (fig. 7). Fig. 5 shows, further that



Fig. 9 Kidney of rat treated daily for six ek th propionyl erythromycin laury sulphate. High lkaline phosphatase activity i the proximal t bules. Frozen section in Formol-CaCl–Macrodex for 10 min., incubation time 30 min. × 400

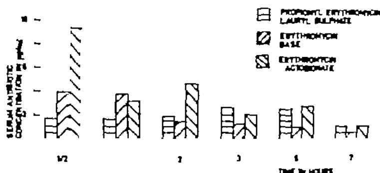


Fig. 10. The average erythromycin concentrations (µg/ml) after single oral doses of propionyl erythromycin lauryl sulphate, erythromycin base and erythromycin lactobionate equivalent to 800 mg erythromycin/kg.

the activity of succinate dehydrogenase was slightly lower in the liver of the PELS group than in the other groups

Figure 8 deals with the kidney studies, similar to those of the liver presented in fig. 5 The kidneys showed neither fat nor glycogen. The activity of glucose-6-phosphatase was higher in the PELS group than in the other groups. Acid phosphatase occurred in relatively large quantities in all the experimental groups. The activity of alkaline phosphatase was highest in the PELS group it occurred in the kidneys primarily in the brush border of the proximal tubules (fig. 9) There were no appreciable differences between the groups with regard to the activity of succinate dehydrogenase.

Skin sections showed no glucose-6-phosphatase. The activity of acid and alkaline phosphatase was highest in the skin of the rats in the PELS group

Serum antibiotic activity

The results for the different groups are given in fig. 10 In the rats treated with EB and EL the peak serum level was reached in half an hour In the rats treated by PELS the peak occurred after 3 hours. After 7 hours, the mean concentration in the serum was almost the same in all groups.

Discussion

In the present test, subchronic administration of erythromycin compounds, EB EL and PELS to rats in large doses killed a number of the

animals. The mortality in each group was significantly higher than that of the control group

The mortality of the animals treated with EB was 50 / of those treated with EL and PELS 33.8 / and of the control animals 16.6 /. Consequently there were no significant differences in mortality between the different experimental groups.

According to KAIPAINEN & FAINE (1954), guinea pigs die quite shortly after the administration of erythromycin. Oral doses of 33–65 mg/kg daily cause the death of nearly 50 / of the test animals after 3 days of treatment. In the present work the rats received the erythromycin compounds in doses about 12–25 times larger and not until after 4 weeks of treatment did animals start dying. According to the literature (MCGUIRE *et al* 1952) mice also tolerate large oral doses during long periods and few test animals die even after such a large single dose as 4000 mg/kg similarly the toxicity of erythromycin is very low for dogs.

The increase in the weight of the test animals of the PELS group was significantly lower than that of the control animals ($p < 0\ 01$) In the other experimental groups too the increase in weight was smaller than among the control animals the differences, however were not significant.

After treatment for 6 weeks the glutamic pyruvic transaminase of the serum in no group exceeded the normal upper limit. The upper limit of the normal was taken to be 350 units/ml (WROBLEWSKI 1958). No group differed significantly from the control group.

Nor did the alkaline phosphatase of the serum in any group rise to a pathological level. However serum alkaline phosphatase in LB group was clearly higher than in the other groups.

Autopsy revealed no distinct pathological tissue changes, a finding that agrees with earlier investigations.

Histologically however changes have been found in the liver and other organs of some test animals both in earlier and in the present studies, although never important enough to account for the death of the animals (KAIPAINEN & FAINE 1954 TIGERTT & GOCHENOUR 1957).

As previously demonstrated histochemically activity of acid phosphatase occurs mainly in the peribiliary areas of rat liver around bile canaliculi. The activity weakens towards the centre of the lobule (WACHSTEIN & MEISEL 1959 NOVIKOFF 1959). Slight activity of alkaline phosphatase is noted mainly in the periportal areas in bile canaliculi, occasionally in the capillaries and the tissue surrounding the bile ducts (WACHSTEIN & MEISEL 1959)

In the present study too, activity of alkaline phosphatase was noted in the connective tissue surrounding the bile ducts and in the arteriolar walls of the liver of the control rats, while a few animals showed negligible

activity in bile canaliculi In contrast, alkaline phosphatase activity was relatively high in the bile canaliculi of some of the rats of the PELS and EL groups

This may be attributable to a slight intrahepatic cholestasis not yet visible and the other stains MEYER & WILLIAMS (1954) showed that the alkaline phophatase in mice, whose hepatic ducts had been ligated 5 days previously was greatly increased in the intrahepatic bile canaliculi, although the other histochemical methods revealed no abnormalities in these livers.

It is interesting to note that the glucose-6-phosphatase activity was highest in the liver of the rats of the PELS group, while the amount of glycogen in their livers was not high (fig. 5). Usually the high glucose-6-phosphatase activity occurs in areas where glycogen has accumulated (NOVIKOFF 1959).

The activity of alkaline phosphatase which in the kidneys is concentrated in the brush borders of proximal tubules (LONGLEY & FISHER 1954 WACHSTEIN 1955 SACHS & DULSKAS 1956 RHODIN 1963), was highest in the animals of the PELS group. This phenomenon is difficult to explain, since alkaline phosphatase activity decreases at least in atrophic tubules (WACHSTEIN 1955) and therefore degeneration of the cells of the tubules cannot be involved.

On the basis of the present investigations we are inclined to believe, like PLANELLES & CHARITINOVA (1964) that the death of the experimental animals is attributable to a change in the intestinal flora released by the antibiotics. This again, in the course of time, leads to disturbed vitamin metabolism and malnutrition of the animals. This view is supported by the retarded weight increase before death and the observation, that the rats treated with EB EL or PELS regularly ate the dead animals if left in the cage. No such cannibalism was noted among the control animals.

An interesting observation was that the fur of the rats treated with PELS was macroscopically seen to have thinned and decreased during the experiment. Microscopic studies then revealed a marked atrophy of hair follicles. Similar observations have not been reported in the literature before The atrophy of the hair follicles is difficult to understand unless avitaminosis produced by the antibiotic is taken to be the primary cause in this case, as the importance of vitamins in the growth of rat hair has already been conclusively demonstrated.

Also bearing in mind that in the PELS group the weight increase was smallest, it seems to us that PELS produces in rats more severe nutritional disturbance than LB and EB which again is responsible for the effects noted.

Summary

In a subchronic toxicity test on rats, propionyl erythromycin lauryl sulphate was found to produce a significantly smaller weight increase than that noted in the control animals, and those treated with erythromycin base or erythromycin lactobionate ($p < 0.01$)

Mortality in all groups was definitely higher than in the control group The cause of death could not be traced by histological and histochemical studies, nor did the surviving animals show any pathologically significant changes in the liver kidneys, adrenals, serum level of glutamic pyruvic transaminase or alkaline phosphatase. Marked atrophy of hair follicles was noted histologically in the rats of the propionyl erythromycin lauryl sulphate group but in no other group

None of these erythromycin compounds could be shown to produce a definite hepatotoxic effect, although amounts of alkaline phosphatase sufficient to suggest incipient intrahepatic cholestasis were demonstrable histochemically in the bile ducts of the animals treated with erythromycin lactobionate and propionyl erythromycin lauryl sulphate.

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Autoradiography

Whit mice weighing approximately 20 grams received 0 57 mg (° μC) of ^{14}C-propranolol hydrochloride in 0.2 ml of physiological saline solution in the tail vein. The animals were sacrificed after 5, 20, and 40 minutes, and 1 4 and 24 hours. Pregnant mice injected with the same amount of radioactive propranolol were sacrificed after 20 minutes and after 4 hours.

In another series of experiments, mice pretreated with 24 mg/kg of phenoxybenzamine i.v were injected 40 minutes later with 0.57 mg (2 μC) of ^{14}C-propranolol hydrochloride. These animals were sacrificed after 20 minutes.

Under ether anesthesia, the mice were quickly frozen by immersion into hexane cooled with solid carbon dioxide. They were then embedded in carboxymethylcellulose and sectioned for whole body autoradiography as described by ULLBERG (1954). Sections twenty μ and 80 μ thick were prepared and dried in a cold room (−10°), then exposed to Structurix x-ray film.

For the study of ^{14}C-propranolol distribution in the brain, male Sprague-Dawley rats, weighing bout 180 g were injected with 1.57 mg (5.5 μC) intravenously and sacrificed after 3 and after 40 min. The brains were quickly removed and frozen on a block of solid carbo dioxide. After embedding in carboxymethylcellulose 80 μ thick frontal sections ere prepared and autoradiographed.

Tissue extractio

Two male albino mice (20 g body weight) were njected i. in the tail vein with 0 57 mg (2 μC) of ^{14}C-pr pranolol hydrochloride dissolved in 0.2 ml of physiological saline solution. One mouse was sacrificed after 20 min. and the other after 40 min. Brain, lung, liver kidney and heart were removed and weighed Each organ was homogenized in an all glass homogenizer together with ml of distilled water After homogenization, the pH was djusted to 9 by the addition of 0.5 N-N OH. The homogenate was shaken for 15 min. with 20 ml of chloroform and centrif ged. When ^{14}C-propranolol was added to tissues before homogenization nd carried through the extraction, more than 98% of the radioactivity was found in the chloroform layer The radioactivity in the chloroform, the water phase, and tissue debris was determined i a Packard liquid scintillation counter One tenth ml each f the chloroform and water phases were counted i mixture of 7 ml 0.5% DPO (2,5-diphenyloxazole) in toluene and 3 ml bsolute ethanol A 15 ml quantity f 0 5% DPO + 0.03% 1.4 bis-2-(4-methyl 5-phenyl oxazolyl) benzene POPOP and 4% Aerosil in toluene was added to the tissue debris (BUHLER 1962 SNYDER & STEPHENS 1962). The hl roform phase was concentrated under stream of nitrogen, and the residue taken for thin-layer chromatography Thin-layer silica gel G_F plates were prepared according to S HL (1962), and developed i ethanol-amm niawater (80 4 5) Radioactivity on the thin-layer plates wa determined first by autoradiography using Kodirex x-ray film for locating the radioactive areas and secondly by scraping the silica from those areas into glass vials and counted in a suspension according to BUHLER (1962)

Results

Distribution studies

Within 5 min. after the intravenous injection of ^{14}C– lol radioactivity was taken up by most tissues and had the bloc to a low level Those tissues accumulating t vi.

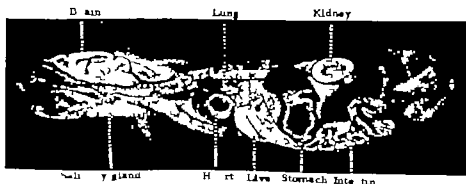


Fig. 1 Autoradiogram showing distribution of radioactivity (light areas) in mouse 5 minutes after intravenous injection of ^{14}C-propranolol. Note high concentration in brain, lung, liver stomach mucosa and kidney

initially were the pituitary and gray matter of the brain, the lung, bronchi, liver kidney and gastric mucosa (fig. 1). The pancreas, spleen, intestines, heart, skeletal muscle, salivary gland, and thymus all accumulated radioactivity but to a lesser extent.

The brain retained high activity in the 5 and 20 minute experiments and the activity then decreased although the cortical gray matter and the hippocampus decreased at a much slower rate (fig. 2) Some activity still remained in the hippocampus 4 hours after the injection (fig. 3). The pituitary was very highly radioactive within the first 5 min. and was still active after 4 hours.

The lungs were very highly labelled and the activity remained quite high even after 4 hours. The bronchi also retained high activity

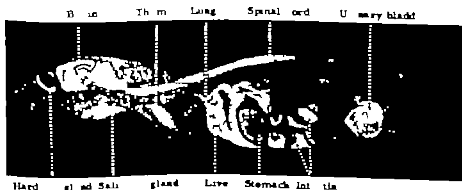


Fig. 2. Distribution of radioactivity in mouse 1 hour after intravenous injection of ^{14}C-propranolol. High concentration is seen in the intestinal content, brain, lung, liver and urinary bladder

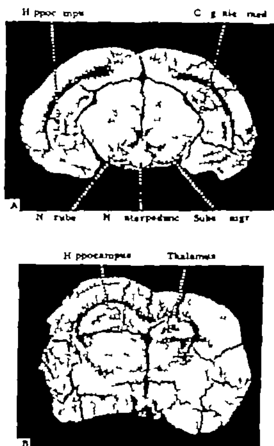


Fig. 4 Autoradiogram showing the distribution of radioactivity (light areas) in rat brain 3 minutes (A) and 40 minutes (B) after intravenous injection of ^{14}C-propranolol.

In the *metencephalon* the areas accumulating the highest radioactivity initially were the nuclei olivaris, tractus spinalis nervi, intercalatus, vestibularis, and cochlearis. After 40 min. the radioactivity decreased significantly in these areas, becoming weak and diffuse

The *mesencephalon* in 3 min. showed a high localization of labelled ^{14}C-propranolol in the nucleus interpeduncularis, substantia nigra, nucleus nervi trochlearis nucleus ruber corpus geniculatum mediale, and the colliculi. There was a general, low level of radioactivity throughout the mesencephalon at 40 min with a slightly higher activity remaining in only the corpus geniculatum mediale and nucleus interpeduncularis.

Moderate to high levels of radioactivity were observed in the *diencephalon* mainly in the thalamus, nucleus caudatus and putamen at 3 min. but decreased to low levels at 40 min At this time the thalamus had slightly higher activity

The *cerebral cortex* was found to take up high radioactivity within 3 min. and was the most active among all the areas at this time. At 40 min., the cortex together with hippocampus and thalamus equally retained the highest level of radioactivity in the CNS. After 4 hours, the hippocampus was still labelled.

Discussion

The distribution of radioactivity after the intravenous injection of ^{14}C propranolol, the recently introduced β-receptor antagonist, was traced in mice by whole-body autoradiography However interpretation of the autoradiogram sections may be complicated by the fact that the radioactivity in the sections may not only represent the injected drug but also the metabolites. To determine the percentage radioactivity representing unchanged propranolol remaining in the tissues, it was considered necessary to use solvent extraction and thin-layer chromatography on selected tissues. As seen in table I even after 40 min., substantial amounts of unchanged ^{14}C propranolol are still present.

Propranolol follows a distribution pattern quite similar to many other organic bases studied in this laboratory Following intravenous injection, ^{14}C-propranolol rapidly cleared from the blood and appeared to be actively taken up by certain tissues. Although the accumulation of radioactivity in an organ may not necessarily indicate pharmacological site of action, the three β-receptor blockers, DCI (Mayer 1962) Kö 592 (Stock & Westermann 1965) and propranolol show a similar rapid uptake and distribution picture, but one which is different from that of the α receptor blocker phenoxybenzamine, (Masuoka *et al.* 1967). Moreover the injection of phenoxybenzamine into a mouse 40 min. earlier did not change the ^{14}C propranolol distribution and binding picture The present study by autoradiography shows that in some of the organs accumulating radioactivity there are certain structures which are particularly active, e.g. germinal centers of the white pulp of the spleen, lymph nodules in the intestines, pancreatic islet tissue, ovarian follicles and an area between the adrenal medulla and cortex. It may be recalled that the α receptor blocker phenoxybenzamine, showed no activity in the white pulp of the spleen but slight activity in the red pulp, as well as some in the corpus lutea and adrenal cortex (Masuoka *et al* 1967).

In view of the potent action of propranolol in blocking the cardiac chronotropic and inotropic effects of catecholamines (Black *et al.* 1964 & 1965) the absence of marked localization in the heart was quite unexpected. This lack of marked localization may possibly be explained as being to the minimal uptake into non-specific sites or storage sites and

fact that the ratio of β-receptor sites to tissue mass is low Recent studies in this laboratory on the subcellular fractionation of rat heart indicate that labelled propranolol may be bound to membrane structures (MASUOKA, ALCARAZ, & EARLE, unpublished).

Pharmacological and clinical studies (LESZKOVSZKY & TARDOS 1965 OWEN & MARSDEN 1965 GRANVILLE-GROSSMAN & TURNER 1966 MUIRMANN *et al* 1966 STEPHEN 1966) have indicated that propranol has no effect on the CNS Indeed the present investigation reveals that labelled propranolol easily passes through blood-brain barrier and is, in fact, accumulated in high concentration in the gray matter The acute toxic effects of these compounds in mice have been attributed to a non-specific action on the central nervous system and not as a result of β-receptor blockade (BLACK *et al.* 1965) On the basis of autoradiography sections of brain prepared in this laboratory showing the distribution of ^{131}I albumin, it appears that the initial 3-minute distribution picture of ^{14}C propranolol may reflect to some extent the vascularity of the various areas (CASSANO *et al.* 1965). However the 40-minute picture indicates that propranolol is taken up and bound by certain structures especially in the gray matter of the cerebral cortex, hippocampus and thalamus. Whether this prolonged binding indicates binding to β-receptors is not known since the presence of such receptors in the brain is still uncertain. There is, however some evidence to indicate that β-receptors may be present in the CNS (GOLDSTEIN & MUNOZ 1961 GAGNON & MELVILLE 1966 SHARE 1966)

Summary

The distribution of ^{14}C propranolol was studied in wholebody sections of mice and brain of rats by autoradiography Radioactivity disappeared from the blood within 5 min. and was taken up extensively by several tissues especially the CNS lung, liver and kidney After 20 and 40 min., *various tissues were extracted with solvent* and it was found that a substantial percentage of the radioactivity remaining in these tissues still represented unchanged propranolol.

In the CNS high radioactivity was localized mainly in the gray matter of rat brain.

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Release of Histamine from Sensitized Rat Peritoneal Cells by Specific and Unspecific Antigens

By

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Histamine is released from peritoneal mast cells or mesentery of sensitized rats on incubation *in vitro* with a specific antigen (horse serum) (UVNÄS & THON 1959 MOTA & ISHII 1960 GARCIA AROCHA 1961 PERERA & MONGAR 1963 NORN 1965). This release is inhibited if the rats are treated with various antirheumatic agents administered towards the end of the sensitization period (NORN 1965).

The object of the present study was to investigate whether this release of histamine takes place only on incubation with a specific antigen, unspecific antigens being ineffective. In that case, the release of histamine would result from a specific antigen-antibody reaction. Moreover the question was investigated whether the antigenic factor in horse serum is a high- or low-molecular substance.

Experiments and Methods

Sera

Serum from horse, x, rabbit, man, and rat was inactivated by heating to 56 for 30 min.

Dialysed horse serum

Horse serum was dialysed against water or modified Tyrode solution (*vide infra*) as f llow 5 00 ml horse serum was put into a dialysis tube (Visking seamless cellulose tubing, average pore radius f 24 Å) placed in cylinder with 20.0 ml modified Tyrode solution or water as outer fluid. The cylinder was gently rocked, and the outer fluid changed 3 times at 4-hourly intervals.

Non-sensitized rat

These rats were female Ibeno rats weighing from 150–200 g.

Sensitized rats

The same type of rats, weighing between 120 and 150 g, were sensitized to horse serum (NORN 1965) or to egg albumin as follows: 10 mg dried egg albumin (not crystallized) dissolved in 1 ml modified Tyrode solution was injected subcutaneously into the rats. At the same time the rats were given intraperitoneally 1.0 ml pertussis vaccine (10^{10} bacteria/ml, Statens Seruminstitut, Copenhagen) cultured on liquid medium without serum. 5 weeks after this treatment the rats were used as the sensitized animals.

PCS

A peritoneal cell suspension (PCS) was removed from sensitized as well as from non-sensitized rats in the following way: 16 ml of 37° modified Tyrode solution (1.44×10^{-1} M NaCl, 3×10^{-3} M KCl, 8×10^{-4} M $CaCl_2$, 8×10^{-3} M Na_2HPO_4, and 3×10^{-3} M KH_2PO_4) was injected intraperitoneally. The animals were killed by exsanguination from the carotid arteries and the peritoneal cavity was opened and emptied of fluid. The cell content of the fluid mainly consisted of lymphocytes, eosinophilic leukocytes, and mast cells (about 2%) (NORN 1967b).

Release of histamine by dialysed and non-dialysed serum

Peritoneal cell suspensions from rats sensitized to horse serum were pooled. From this pool samples of 3.90 ml were removed. To each sample 250 µl horse serum was added, either non-dialysed or dialysed against water or modified Tyrode solution. Corresponding samples were made up with 250 µl of the outer fluids from the first dialysis of horse serum – or prepared with the same volume of water or of modified Tyrode solution (blank samples). All the samples were incubated for 30 min. at 37°. Thereafter the quantity of histamine liberated was determined as per cent of the total content (NORN 1967), no correction being made of the small quantity of histamine which was liberated by the mechanical manipulation.

Histamine release by specific and unspecific antigen

Corresponding samples were incubated, as already mentioned, with horse serum (specific antigen) or with serum from ox, rabbit, man, or rat (unspecific antigen). As blank samples the author used partly the same kind of samples incubated with modified Tyrode solution instead of serum and partly samples of PCS from non-sensitized rats, incubated with horse serum or modified Tyrode solution. The histamine release is given in table 2.

Similar experiments were carried out on samples of PCS from rats sensitized to egg albumin (cf. table 2). As specific antigen 250 µl 1.5% egg albumin (5 × crystallized) was used.

Results

Release of histamine by dialysed and non-dialysed serum

On incubation of PCS from rats sensitized to horse serum with dialyzed horse serum the same percentage of the *total histamine content* of the sample (about 42%) is released as on incubation with non-dialysed horse serum (table 1). This applies when this serum has been dialysed against modified Tyrode solution or against water. Incubation of corresponding PCS samples with the respective outer fluids from the first dialysis of

Table 1

Release of histamine in peritoneal cell suspensions (PCS) from rats sensitized to horse serum by dialysed and no -dialysed horse serum, outer fluids before and after first dialysis of horse serum (inner fluid) Histamine release given in per cent of total content. Duplicate determinations.

PCS incubated with

horse serum			outer fluid fter dialysis		outer fluid before dialysis	
non-dialysed	dialysed against mod. Tyrode	dialysed against water	mod. Tyrode	water	mod. Tyrode	water
43	41	49	2	4	4	4
41	41	44	1	2	2	3

horse serum does not result in the release of more histamine than does incubation with modified Tyrode solution or with water (blank samples), in which case 1–4 / of the total histamine content is released.

Table 2

Release f histamine in peritoneal cell suspensions from sensitized and non-sensitized rats by sera, egg albumin nd modified Tyrode solution. Duplicate determinations.

PCS from rats which were	PCS incubated with	Histamine liberated (per cent of total)	
non-sensitized.	mod. Tyrode	2	3
	horse serum	1	3
	egg albumin	4	3
sensitized t horse serum	horse serum	53	48
	bovine serum	2	3
	rabbit	1	1
	humane	1	2
	rat	1	3
	mod. Tyrode	3	3
sensitized t egg albumin	egg albumin	46	48
	horse serum	2	3
	mod. Tyrode	3	3

Release of histamine by specific and unspecific antigen

Table 2 shows that in PCS from rats sensitized to horse serum, about half the total histamine content of the sample is released on incubation with horse serum. Serum from ox, rabbit, man or rat does not release more histamine in PCS than does modified Tyrode solution (blank sample), in which about 3% of the total histamine content is released. The same applies if PCS from non-sensitized rats is incubated with horse serum or with modified Tyrode solution.

The table also shows how much histamine is released from samples of PCS from rats sensitized to egg albumin, when these samples are incubated with egg albumin, horse serum, or modified Tyrode solution. The egg albumin release about half the total histamine content of the sample, while horse serum and modified Tyrode solution release only about 3%. If PCS from non-sensitized rats is incubated with egg albumin, the released quantity is also about 3% of the total histamine content.

Discussion

In peritoneal cell suspensions from rats sensitized to horse serum about half the intracellular histamine content of the cells is released on incubation with horse serum. On the other hand, practically no histamine is released on incubation of the cell suspension with other antigens such as bovine, human, rabbit, or rat serum. In such cases the release is less than 3% of the total histamine content, and thus must be due to the mechanical manipulation of the cells, since this is the same percentage which is released when the cell suspension is incubated with modified Tyrode solution instead of serum (blank sample). The same applies when suspensions of peritoneal cells from rats sensitized to egg albumin are incubated with egg albumin or with horse serum. Egg albumin releases 46% of the total histamine content, while horse serum releases only 2%.

On incubation of peritoneal cell suspensions from non-sensitized rats with horse serum or with egg albumin, no more histamine is released (about 3%) than on corresponding incubation with modified Tyrode solution. In other words, the peritoneal cells from non-sensitized animals are insensitive to these antigens as are corresponding cells from sensitized animals to unspecific antigens.

The experiments show that specific antigen releases histamine from peritoneal cells of sensitized rats, while unspecific antigen releases no histamine. Thus, the release of histamine is caused by a specific antigen-antibody reaction.

Dialysed horse serum releases just as much histamine in the peritoneal

cell suspension from rats sensitized to horse serum as non-dialysed horse serum. The dialysate has no histamine liberating effect. Thus, since the entire histamine-releasing activity is found in the dialysed serum, it must be due to high-molecular substances, presumably plasma proteins.

Summary

The release of histamine was studied in suspensions of peritoneal cells from sensitized rats following *in vitro* incubation with specific or unspecific antigen. Specific antigen (horse serum or egg albumin) releases histamine from the peritoneal cells, while unspecific antigen has no histamine liberating effect. Thus, the release of histamine is effected by a specific antigen antibody reaction.

The question was investigated whether horse serum loses its histamine releasing activity after dialysis. As this is not so, the histamine-liberating activity must be due to high-molecular substances, presumably plasma proteins.

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The Stability of Noradrenaline in Infusion Solutions

By

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Noradrenaline (NA) and adrenaline (A) are often administered therapeutically as "drop" infusions during several hours. The catecholamines are dissolved in different solutions containing e.g. 0.9 % sodium chloride or 5.5 % glucose. The stability of the catecholamines in these solutions must be taken into account since these compounds are readily oxidized in certain conditions (see e.g. VON EULER 1956). Factors which favour the inactivation of these compounds are e.g. alkaline milieu, high temperature, and the presence of heavy metal ions. WEST (1952) found that the activity of NA in distilled water, in 5 % dextrose solution, and in isotonic sodium chloride was reduced about 40–60 % when the solutions were stored for 48 hours at 18°. When the solutions were stored at 37° the loss was about 80-95 %. The rate of destruction was greater when the solutions were stored at a higher pH.

Thus it seems possible that NA, when used therapeutically, can be destroyed during its storage in the infusion bottle. In fact some drug companies advise that NA should not be added to bicarbonate solutions. In order to elucidate this problem we have determined the contents of NA in different infusion solutions at different times after the drug has been added to the solution.

Methods

The studies were performed using l-noradrenaline bitartrate or nor-exadrin ® conc. (Astra). An ampoule of nor-exadrin ® conc. contains per ml 1 mg NA, 7 mg sodium chloride, 1 mg pyrosulphite, 1 mg metazin, tartaric acid, and distilled water to make 1 ml. Four mg NA (two ampoules nor-exadrin ® conc.) were diluted i 1000 ml sterile sodium chloride solution (0.9 %), glucose solution (5.5 %) or 900 ml sterile sodium bicarbonate solution (1.4 %). The pH values of the sodium chloride, glucose solutions, and the bicarbonate solution were 6.5, 5.0, and 7.9, respectively. An infusion apparatus was coupled

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From The Department of Pharmacology University of Copenhagen
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Paper Chromatographic Evidence of the Elimination of ^{14}C Decamethonium as the Unchanged Compound in the Rabbit

By

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A study of the renal excretion following intravenous injection of ^{14}C decamethonium in rabbits has shown that approximately ¾ of the compound is excreted within 24 hours (BROEN CHRISTENSEN 1966) Paper chromatography of samples of urine showed that decamethonium is ex creted in an unchanged form within the first two hours after injection, but chromatography of samples collected later however gave the impression that a metabolite was excreted. A ^{14}C-decamethonium compound with a relatively low specific activity was used and hence the chromatographic studies of samples with a low content of decamethonium were subject to considerable uncertainty

In studies on the fate of ^{14}C-decamethonium after intravenous injection into cats (WASER & LÜTHI 1965) it was possible to demonstrate a slight amount of a non-identified metabolite in an aqueous extract of liver tissue, whereas chromatography of plasma, muscle extract and urine showed only unchanged decamethonium within the first hour after injection. ^{14}C-decamethonium with a high specific activity was used for these studies.

The object of this study has therefore been to see if it is possible, in a more thorough investigation, to demonstrate a metabolite of decamethonium after intravenous injection into rabbits of a ^{14}C-labelled compound with a high specific activity In addition a more sensitive method has been developed for scanning the radioactivity on the chromatogram this is described in the following.

Methods

Experimental technique

12 albino rabbits weighing from 1930 to 3150 g were used for the study A single dose of 90 μg ^{14}C-decamethonium dibromide per kg was used in all the experiments and injected into the marginal ear vein using a carrier free 0.9 % solution of NaCl The ^{14}C-decamethonium was produced by The Radiochemical Centre Amersham, England, with a specific activity of 139 μc/mg. The radiochemical purity of the compound was verified by paper chromatography at the beginning and end of the experimental series. It was found that the preparation corresponded to the given specification more than 97 % of the activity resulting from ^{14}C-decamethonium.

The methods in general have been described previously (BROEN CHRISTENSEN 1966). In 9 short term experiments, a cannula was introduced into the trachea and polythene catheter into the carotid artery while under halothane-N_2O-O_2 anaesthesia. The object of this was t register the respiration and blood pressure. A trans-urethral catheter was also introduced int the bladder for fractional urine collection in 4 of the animals. At the end of the experiments, i.e. 1–4 hours after the decamethonium injection, the animals were killed by intravenous insufflation of air and samples from th liver and the femoral extensor quadriceps muscle were removed for chromatographic study The pH of the urine samples were measured by glass electrodes and a pH-meter 25 (Radiometer Copenhagen)

In 3 long-term experiments on intact animals the 24 hour urine was collected after the injection of decamethonium, whilst the animals were kept in a metabolism cage.

The measurement f the radioactivity in the urine samples was carried out as previously described (BROEN CHRISTENSEN 1966) by the liquid scintillation technique using a Packard Tri-Carb Scintillation spectrometer model 3003

Th chromatographic technique

Chromatography of urine, organ extracts and preparation injected was carried out by an ascending technique on Whatman paper no. 1 Two different systems were used as the mobile phase A, n-butanol, ethanol, glacial acetic acid, water (8 2 1 3) and B, ethanol, water ammonia (75 25 2). The technique has been described in detail in a previous paper (BROEN CHRISTENSEN 1966).

The chromatography of the urine samples was carried out both on undiluted urine and on an extract of urine containing equal parts f acid alcohol (ethanol, glacial acetic acid and water 10 1 3 v/v/v). 5 to 10 μg of non-labelled decamethonium dibromide dissolved in 100 μl of water was used as the control.

Approximately 1 g of tissue was homogenized in 5 ml 80 % methanol and centrifuged. The resulting supernatant fluid was evaporated and the residue gain dissolved in 200 μl of water which was applied to the chromatography paper Non-labelled decamethonium added to the tissue extract from a control animal, in amounts allowing the demonstratio of reference spot o the paper by Dragendorff's reagent, was used as reference.

Two methods were used for scanning the ^{14}C-radioactivity on the chromatograms, first an utomatic chromatogram scanner (model Philips) secondly more sensitive method based on liquid scintillation technique. This was used because of the relatively low amounts of activity in the organ extracts (BOUSQUET & CHRISTIAN 1960). A strip of the chromatogram with width f 2 cm was cut into rectangular pieces (2 × 1 cm or 2 × ½ cm). The pieces of paper were placed at the bottom f counting vial in 4 ml of scintillation medium (BRAY 1961) The addition of concentrated hydrochloric acid to the scintillation medium (100 μl concentrated HCl to 60 ml scintillation medium, see HERBERG 1958) was

necessary in order to avoid spurious counts due to phosphorescence. The results of the measurements of the activity were represented graphically for comparison with the reference spot (fig. 1).

Repeat counting of the samples after shaking the counting vials showed that the position of the paper at the bottom of the counting vial had no noteworthy influence on the count. Thus 6 consecutive measurements of samples corresponding to maximum activity in fig. 1 showed a standard deviation of 7.5 % of the mean value.

Results

It was possible to recover an average of 85 % (range 78–89 %) of the injected dose following measurement of the radioactivity in the urine in the 3 experiments in which the urine was collected for the first 24 hours after the injection. In 4 tests, in which the urine was collected for the first 4 hours after the injection, an average of 54 % of the dose was recovered (range 42–71 %).

Measurement of the radioactivity in the urine samples before and after centrifugation showed that a varying amount of the activity could be precipitated. This finding was most marked in the samples from the long-term experiments, where up to [illegible] of the radioactivity could be precipitated by centrifugation.

Chromatography of the urine samples

Chromatography of freshly passed, undiluted urine from the long term experiments gave R_F-values for ^{14}C which were very near the R_F-value for authentic decamethonium, however the chromatograms showed some tailing.

Chromatography with solvent A (n-butanol, ethanol, glacial acetic acid and water 8 : 2 : 1 : 3) of urine samples collected from animals subjected to anaesthesia, at times clearly showed two peaks of activity in the chromatogram, one corresponding to the authentic decamethonium and another with a higher R_F-value (fig. 1). This second spot was particularly well seen in two samples from the period 2 to 4 hours after the injection, where the pH of the urine was respectively 5.8 and 6.2 (in all the remaining samples the pH was between 6.7 and 8.0).

When 5–10 μg of non-labelled decamethonium were added to the samples, it was possible with Dragendorff's reagent to demonstrate this on the chromatogram in the same localization as the radioactivity. This was also the case where the chromatogram contained two radioactivity spots.

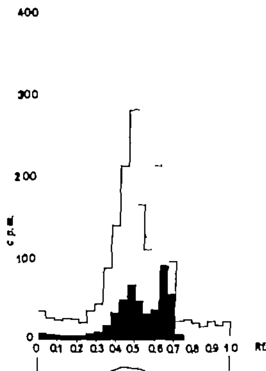


Fig. 1 Scanning results from paper chromatography of urine samples collected 120–180 minutes after intravenous injection of 90 µg/kg ^{14}C-decamethonium. Solvent system n-butanol, ethanol, glacial acetic acid, water (8 2 1 3). Distribution of ^{14}C-activity detected by liquid scintillation (upper broken line) compared with the result obtained with an automatic chromatogram scanner (black columns) Abscissa R_f-value. Ordinate Counts/minute. At the bottom Control chromatography of authentic decamethonium stained with Dragendorff' reagent.

Chromatography of the extract of the urine with acid alcohol

As mentioned above a varying amount of the radioactivity could be precipitated by centrifugation of the urine samples. An attempt was made to find a solvent with which the total activity could be extracted from the samples. The results of the extraction by shaking the urine with equal parts of different solutions are shown in table I

Following these tests, acid alcohol was chosen as the extraction solution. It was only possible to demonstrate traces of radioactivity in the organic phase after shaking the urine samples with n-butanol and 1.2 dichlorethane.

Chromatography of extracts of all the urine samples after shaking with

Table 1

Extraction of ^{14}C-activity in urine with different solvents.

To the urine samples equal volumes of solvent were added. This was shaken mechanically and then centrifuged for 10 minutes at 3000 r.p.m. Extraction of ^{14}C-activity is expressed as counting-rate for the supernatant in percent of counting rate for the mixture before centrifugation.

Solvent	H_2O	1/10 mol $BaCl_2$	Methanol	Ethanol	Ethanol, glacial acetic acid, water (10:1:3)
Percentage extraction	49	77	78	86	96*

*) Result from a single experiment. The average from all experiments (7) was 92 ± 5 % (M ± SD).

acid alcohol gave the same R_f-values for the radioactivity as the authentic decamethonium with both solvents, and in no case was there more than one peak of radioactivity. The results of two experiments are shown in table 2. In one experiment urine samples were used which had shown two peaks of activity in the chromatography of the untreated sample (fig. 1) and in the other a sample from a long-term experiment containing approximately 85 % of the injected dose was used.

Table 2

Paper chromatographic identification of ^{14}C-radioactivity in extracts of urine with acid alcohol after intravenous injection of ^{14}C-decamethonium.

^{14}C-radioactivity was detected by liquid scintillation technique. Controls consisted of authentic decamethonium.

Sample	Solvent system	R_f-value	
		Control	^{14}C
Urine collected 120–180 min. after injection (4-hours experiment)	A	0.47	0.48
	B	0.62	0.64
Urine collected 0–24 hours after injection (long-term experiment)	A	0.45	0.45
	B	0.67	0.67

*) Solvent systems were A, n-butanol, ethanol, glacial acetic acid and water (8:2:1:3) and B, ethanol, water and ammonia (75:23:2).

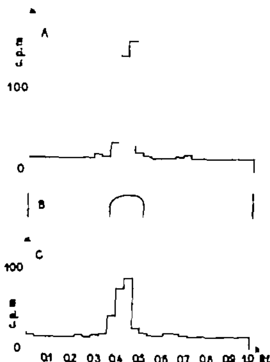


Fig. 2. Scanning results and chromatogram from paper chromatography of methanol extract of tissues. Solvent system n-butanol, ethanol glacial acetic acid and water (8:2 1 3). The activity was detected by liquid scintillation. The control spot was developed by Dragendorff's reagent. Samples were taken 60 minutes after intravenous injection of 90 µg/kg ^{14}C-decamethonium. A. Extract of liver homogenate. B. Authentic decamethonium added to muscle homogenate from control animal. C. Extract of muscle homogenate (from the same animal as A.).

Chromatography of the organ extracts

Samples were taken from a total of 6 animals from the liver and striated muscles respectively 1 2 and 4 hours after the injection of decamethonium. These were subjected to chromatography with both solvent A and B The results from one of the experiments are shown in fig. 2, together with the chromatogram for the authentic decamethonium. It can be seen from the figure that ^{14}C radioactivity has the same R_f-value as authentic decamethonium.

Discussion

The results of the ^{14}C measurements of the urine samples collected in the 24 hours after the decamethonium injection are in agreement with the results obtained from the use of a preparation of low specific activity

No support has been found for the suspected renal excretion of a metabolite, starting some hours after intravenous administration of decamethonium into rabbits (BROEN CHRISTENSEN 1966). The study has shown that more than 80 / of the injected dose is excreted as decamethonium. In actual fact some of the decamethonium is bound to a non-identified component of the urine. This binding can also be observed *in vitro* and unchanged decamethonium can be liberated from this by extraction with acid alcohol The maximum activity with a R_f-value higher than that for decamethonium shown in fig. 1 must presumably result from a complex containing decamethonium, inasmuch as the extraction from the spot with acid alcohol liberates a ^{14}C labelled compound which is chromatographically identical with decamethonium. The complex is poorly soluble in water

The study of the fate of decamethonium after intravenous injection into cats (LÜTHI & WASER 1965) has shown that up to 50 / of the dose is excreted in an unchanged form in the urine within the first hour after injection. Lüthi & Waser suggested however that part of the decamethonium is bound to the proteins in the bladder mucosa.

It is possible that mucin secreted from the epithelium of the urinary tract binds decamethonium. Studies of other quaternary ammonium compounds suggest that the onium ion under physiological conditions can be bound by acid mucopolysaccharides. Thus *in vivo* studies on rats have shown that mucin like hyaluronic acid and chondroitin sulphate can inhibit the absorption of the onium ion from the jejunum (LEVINE & PELIKAN 1961 LEVINE 1961).

It is known from *in vitro* studies that onium ion at a physiological pH can be bound by acid mucopolysaccharides and thus form poorly soluble complexes (SCOTT 1955).

Our chromatographic studies of liver and muscle extracts support the view that decamethonium is not metabolized in the organism. The slight amount of radioactivity (in fig. 2 1–2 / of the total amount) which does not have the same R_f-value as authentic decamethonium, can hardly be considered as consisting of metabolites, as approximately 2 / of the ^{14}C-radioactivity of the compound injected as mentioned above does not result from the decamethonium.

Summary

Paper chromatographic studies of the urine, extracts of liver and muscle tissue from rabbits which have received 90 µg/kg decamethonium intravenously give no support to the assumption that decamethonium is metabolized in the organism.

Within 24 hours more than 80 / of the dose is excreted in an unchanged form in the urine.

In the urine a varying amount of decamethonium is bound to an unidentified component of urine from which it is liberated by extraction with acid alcohol.

Acknowledgements

The Packard Tri-Carb Scintillation spectrometer of the department and the [14]C-decamethonium with the high specific activity used in the study have been purchased with a grant given by Danish State Research Foundation (A 5/65) Support has also been received from the Foundation for the advancement of Medical Science, Copenhagen, for technical assistance which was carried out by Mrs. Karin Jorgen-Jensen.

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Urinary and Gastrointestinal Excretion of Metabolites of Labelled 5-Hydroxytryptamine and 5-Hydroxytryptophan

By

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It is well known that there are species-specific differences in the metabolism of 5-hydroxytryptamin (5HT) (NAKAI 1958 McISAAC & PAGE 1959 AIRAKSINEN 1963) After parenteral administration of labelled 5HT some radioactivity has been found in the faeces of the rabbit (McISAAC & PAGE 1959) and rat (KEGLEVIĆ *et al* 1960) In the rat this was partly due to 5HT-metabolites excreted in the bile (AIRAKSINEN 1963) In the present paper the metabolites of labelled 5HT and 5-hydroxytryptophan (5HTP) were studied in the cat and rabbit representing a carnivorous and a herbivorous species. In addition to urine and bile the gastric and intestinal (+ pancreatic) juices were also analysed. The effect of a 5HT antagonist (methysergid) a 5HT releaser (reserpine) and an amine oxidase inhibitor (pargyline) on the gastric excretion of the metabolites of labelled 5HT was studied in rats.

Methods

Male white rabbits eighing from 2.4 t 2.6 kg and male cats of the weighing 1 9 t 3.6 kg were kept without solid food for three days before the experiment. They were llowed to drink milk ad libitum during the first tw days and only water d ring the last day The rabbits were anaesthetized with urethane (1 7 mg/kg intravenously) and the cat were induced w th ether nd then given chloralose (80 mg/kg intravenously) The nimals were tracheotomized and then given artificial respiration. A cannula for the infusion of the drug and Ringer solution as inserted into jugular ein The bdomen wa opened, the pylorus ligated and the urinary bladder stomach and biliary duc t were cannulated plastic tubes Aborally from the papilla of Vater the duoden m was ligated and 15–20 cm further dow the intestine jejunum was closed by another ligature and plastic tubes were inserted into the duodenal nd jejunal lumen bove these ligatures I rabbits the d

cannula collected mainly pancreatic juice, but in cats it was mainly duodenal secretion, since the main pancreatic duct in cats usually runs down into the jejunum. When all the tubes were well inserted the abdomen was closed

5-hydroxytryptamine-3-^{14}C creatinine sulphate (dosage 10 µc/kg + carrier to 10 mg 5HT base/kg) or D L-5-hydroxytryptophan-3-^{14}C (Radiochemical Centre, Amersham) (10 µc/kg, 25 mg/kg) in saline was infused for one hour Samples of gastric, duodenal and jejunal juices as well as bile and urine were collected in 3 8 and 24 hours samples from the start of the drug infusion. The loss of fluids was corrected with Ringer solution, as required.

The rats were anaesthetized with ether The pylorus was ligated and then the abdomen was closed and the rat allowed to wake up (SHAY *et al.* 1954). Then ^{14}C 5HT (20 µc/kg, 10 mg base/kg) was administered subcutaneously After 4 hours the rats were decapitated, the stomach was removed and its contents including the easily removable mucus taken for analysis.

A portion of the samples was extracted with 5 volumes of acetone and the sediment re-extracted with water Both acetone extract, water extract and original samples were used for paper chromatography and after drying on planchetes the radioactivity determined as in previous studies (AIRAKSINEN 1963). Ascending chromatography in butanol-acetic acid-water (4 1 5) and isopropanol-ammonia-water (10 1 1) was used. Indolic spots were visualized by spraying with p-dimethylaminobenzaldehyde reagent. For the localization of the radiactive spots, autoradiograms (chromatoradiograms) were developed on X-ray film (Kodak Blue Rand). One-dimensional chromatograms were also counted directly on paper (AIRAKSINEN 1963)

Results

The excretion of radioactivity after the administration of labelled 5HT and 5HTP in cats and rabbits are shown in tables 1 and 2. The total urinary excretion of labelled compounds was somewhat similar in rabbits and cats. All gastrointestinal excretions after ^{14}C 5HT infusion were lower in cats than in rabbits per time unit. This difference was small per ml in gastric juice, because the volumes excreted were smaller in the cat than in the rabbit. There was only little pancreatic and intestinal excretion of the metabolites of ^{14}C 5HT in both species but slightly more was excreted after the administration of ^{14}C 5HTP

There were considerable qualitative differences in the metabolites of ^{14}C 5HT between the two species. The cat does not form O-glucuronides of 5HT 5-hydroxytryptophol and 5HIAA to any great extent although these are found in the rabbit (fig. 1) The corresponding O-sulphates are major metabolites in cats (fig. 2 and 3) In the urine 5HIAA is one of the major metabolites in both species but in the digestive secretions only traces of 5HIAA were found. Very small amounts of unconjugated ^{14}C 5HT were present in the gastric juice of cats, rabbits and rats (fig. 4) and there was none in the bile. All the radioactivity in the bile and nearly all that in the gastric juice after 5HT administration seemed to be in the form of conjugates of 5HT 5-hydroxytryptophol and 5HIAA.

Table 1

Excretion of radioactivity I 5 anaesthetized cats and 2 rabbits after intravenous infusi f [1] C 5HT (10 μc/kg, 10 mg/kg). For the cats means ± SE are given.

		Cat			Rabbit					
		Cumulative mean vol ml	Radioactivity excreted: % of Dose Cumulative	Radioactivity excreted: μmoles/ml	Cumulative volume ml		Radioactivity excreted: % of Dose Cumulative		Radioactivity excreted: μmoles/ml	
Urine	3 hr	1.58	4.60 ± 2.79	3.85 ± 1.85	7.6,	0	0.02,	–	0.003	–
	8	13.98	16.90 ± 9.26	1.00 ± 0.25	16.3	8.2	4.98	5.35	0.78,	0.90
	24	38.86	20.60 ± 11.30	0.37 ± 0.24	34.5	16.2 *)	26.98	9.49 *)	1.64,	0.70 *)
Bile	3	1.66	0.21 ± 0.11	0.14 ± 0.07	3.9	5.9	0.24,	1.58	0.08,	0.36
	8	3.24	0.49 ± 0.11	0.41 ± 0.14	8.0,	13.7	0.69	3.06	0.16,	0.26
	24	6.52	0.78 ± 0.15	0.16 ± 0.06	16.4,	27.2 *)	1.00,	4.42)	0.05	0.14
Gastric juice	3	2.10	0.007 ± 0.002	0.010 ± 0.003	5.9	20.8	0.03	0.44	0.01	0.03
	8	6.34	0.027 ± 0.016	0.007 ± 0.002	9.8,	24.3	0.05,	0.59	0.01	0.06
	4	10.66	0.072 ± 0.015	0.020 ± 0.013	39.8,	28.5 *)	0.07	0.78)	0.001	0.06)
Duodenal juice	3	0.14	0	0	1.8,	3.1	0.004	0.004	0.003	0.002
	8	0.20	0.004 ± 0.001	0.002 ± 0.001	5.0,	7.8	0.06,	0.10	0.02,	0.04
	24	4.32 *)	0.040 ± 0.027	(0.03 ± 0.02)	13.2,	18.0 *)	0.12,	0.15)	0.009	0.007 *)
Jejunal juice (part only)	3	1.20)	0.015 ± 0.009	(0.02 ± 0.02)	0.2,	0.7	0.01	0.04	0.03	0.07
	8	3.20 *)	0.040 ± 0.039	(0.01 ± 0.01)	1.1	2.5	0.02,	0.18	0.03	0.14
	24	5.52 *)	0.051 ± 0.043	(0.01 ± 0.01)	12.7	2.9)	0.14,	0.19 *)	0.01	0.05

*) 19 hr) Perfusion fluid included.

Table 2

Excretion of radioactivity after intravenous infusion of ^{14}C-d,l 5HTP (10 μc/kg, 25 mg/kg) into two rabbits (two first values) and a cat (last value) during anaesthesia.

		Cumulative volume ml			Radioactivity Excreted % of Dose Cumulative			Radioactivity Excreted μmoles/ml		
Urine	3 hr	2.8	1.8	0.6	4.02	0.89	1.21	3.91	1.35	5.50
–	8 –	20.1	8.0	14.4	37.92	9.62	19.86	5.10	3.93	3.68
–	24 –	30.0	19.2	36.6	53.39	27.25	32.80	4.26	3.89	1.58
Bile	3 hr	8.2	4.8	2.2	0.93	0.48	0.39	0.31	0.28	0.49
–	8 –	19.4	16.8	5.3	2.08	1.53	0.84	0.28	0.25	0.41
–	24 –	34.4	41.4	15.5	2.85	2.36	1.40	0.11	0.09	0.15
Gastric juice	3 hr	11.5	0.8	1.4	0.10	0.03	0	0.03	0.09	0
– –	8 –	49.6	27.3	5.0	0.34	3.55	0.03	0.02	0.37	0.02
– –	24 –	71.3	66.1	10.6	2.27	3.70	0.03	0.24	0.01	0.01
Duodenal juice	3 hr	3.7	2.3	0.1	0.23	0.02	0	0.17	0.03	0
– –	8 –	10.6	6.5	0.1	0.34	0.06	0	0.04	0.02	0
– –	24 –	17.8	20.5	0.2	0.41	0.06	0	0.03	0	0
Jejunal juice	3 hr	0..	0	0	0	0		0	0	
– –	8 –	0.4	0.3	–	0.02	0.00		0,24	0	–
– –	24 –	0.7	0.3	–	0.04	0.01		0.16	0.01	–

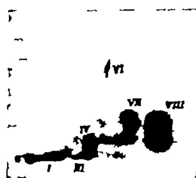


Fig. 1 Chromatoradiogram of rabbit urine 8–24 hr after intravenous dministration of ^{14}C 5HT First run (from bottom to t p) in isopropanol-ammonia-water second run (from left t right) in butanol-acetic acid-water Spots and the corresponding compounds in this and other pectures I 5HT-O-glucuronide, II 5HT-O-sulphate, III 5HIAA-O-sulphate, IV 5-hydroxytryptoph l-O-glucuronide and 5HIAA-O-glucuronide, V 5-hydroxytryptophol-O-sulphate, VI 5HT VII unidentified (rabbit-typical) spot, VIII 5HIAA.



Fig. 2. Chromatoradiogram of urine of cat 3–8 h (left) and 8–24 hr (right) after 14C 5HT administration. For other explanation see the legend of fig. 1

After the administration of 5HTP one of the major spots in urine chromatograms was 5HT It constituted a smaller amount of the radioactivity in the secretion of the alimentary tract. In gastric, duodenal and intestinal juices the main part of the radioactivity consisted of non-metabolized 5HTP In the bile as well as in urine the pattern of the metabolites of 14C 5HTP was more like that of 14C 5HT though non metabolized 5HT and in rabbits, a spot assumed to be 5HTP-O-glucuronide were also found

Table 3 shows the effect of methysergide, reserpine and pheniprazine pretreatments on the gastric excretion of total 14C 5HT metabolites in pylorus-ligated rats. In the control rats, the gastric excretion of radio-



Fig. 3 Chromatoradiogram of bile of cat 3–8 hr after 14C 5HT administration. For other explanations see the legend of fig 1

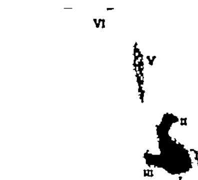


Fig. 4 Chromatoradiogram of the acetone extract of rat gastric juice 0–4 hr after ^{14}C-5HT administration. First run (isopropanol-ammonia-water) from bottom to top, second run (butanol-acetic-acid-water) from right to left. For explanation of the spots see legend of fig. 1 Note that nearly all the radioactivity is in the form of conjugates although much of the glucuronides is not taken up by the acetone extract.

activity during 4 hr was higher than in the anaesthetized rabbits and cats. Methysergide in the same dose as 5HT did not produce any significant changes in the excretion. Reserpinization, on the other hand, markedly decreased the gastric excretion of the radioactivity and the amount of

Table 3

Effect of some drugs on the gastric fluid volume, gastric excretion of labelled 5HT and its content in the gastric wall in four hours after i administration of ^{14}C 5HT (20 μc/kg, 10 mg/kg) in young rats. Number of animals in brackets. Methysergide and reserpine were administered 2 hr phenlprazine 2.5 mg/kg 24 hr and 2.5 mg/kg 2 hr before the injection f ^{14}C 5HT

Pretreatment	Mean volume	Radioactive substances in gastric fluid: % of dose	mmoles/ml	acetone extractable % of total	Radioactive substances in gastric wall % of dose
None (7)	2.9	2.47 ± 1.12	1.03 ± 0.39	78.1	0.39 ± 0.09
Methysergide 10 mg/kg (5)	4.0	2.81 ± 1.35	1.02 ± 0.48	79.9	0.34 ± 0.09
Reserpine 5 mg/kg (5)	2.	0.30 ± 0.09	0.18 ± 0.05	59.4	0.07 ± 0.04
Pheniprazine 5 mg/kg (4)	1.8	1.11 ± 1.48	0.77 ± 0.15	74.8	0.12 ± 0.06
Pheniprazine + reserpine (4)	1.8	0.58 ± 0.34	0.70 ± 0.36	76.0	0.19 ± 0.08

labelled compounds in the gastric wall 4 hr after the [14]C 5HT administration. Pheniprazine tended to decrease the radioactivity of gastric juice and wall when expressed as per cent of the dose, but also produced a decrease in the gastric fluid volume, and the radioactivity per ml showed only a nonsignificant decrease ($p > 0.05$). Pheniprazine, however blocked the inhibitory effects of reserpine on the excretion of radioactive compounds and gastric fluid volume.

Reserpine decreased the acetone-extractable fraction of the radioactivity in gastric juice In reserpinized rats, the glucuronides of 5-hydroxyindoles formed nearly all the radioactivity excreted. Pheniprazine decreased the amount of 5HIAA and its conjugates. Methysergide had no effect on the quality of the metabolites of 5HT in gastric juice.

Discussion

The results indicate that metabolites of 5HT are excreted into the gut contents mainly in the bile and gastric juice while little is found in the pancreatic and intestinal juices. The gastric and intestinal excretion may be higher when the animals have food in the lumen, as the wall distension of the digestive channel (BÜLBRING & CREMA 1959 KÄRKI *et al* 1960) as well as salts and H ions (ERSPAMER & BERTACCINI 1962) can release 5HT both into the portal blood and gut lumen. But the physiological 5HT release into the gut contents probably originates mainly in the mucosal enterochromaffine cells and not in the blood. This is not revealed by the study involving 5HT infused into the circulation but, however experiments with 5HTP may reflect partly the decarboxylation in the gastrointestinal wall Radioautographic studies show that 5HTP is taken up better than 5HT by the exocrine cell groups of pancreas and mucosal enterochromaffine like cells of the stomach of rats (RITZÉN *et al* 1965). In our study however much of the radioactivity in the gastrointestinal secretion after [14]C 5HTP infusion was not metabolized and the lower excretion after [14]C 5HT administration can be explained partly as a result of its effect on the gastrointestinal circulation and the decrease in secretion volumes.

The gastric excretion of 5HT does not appear to be merely a passive diffusion since it is inhibited by reserpine. Probably 5HT is actively taken up by some kind of mucosal cells that can metabolize 5HT and release the metabolites into the gastric juice. Reserpine can inhibit this by preventing the 5HT uptake into mucosal cells. It is not known if these 5HT metabolizing and excretory cells in the gastric mucosa are enterochromaffine or other types of cells.



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The gastric excretion of ^{14}C 5HT metabolites is not correlated with the secretion of H^{+} ions or pepsinogen (MATTILA, HUTTUNEN & AIRAKSINEN, unpublished). Since 5HT has been reported to increase the gastric mucus in some mammalian species (GARATTINI & VALZELLI 1965) this suggests that 5HT is taken up by the mucous cells and that its metabolites are excreted in the mucus. In our study in Shay rats 5HT increased only the relative amounts of mucus components (hexoses, hexosamine, sialic acid) in gastric juice while reserpine was shown to inhibit the gastric mucus production as it inhibited the gastric excretion of 5HT metabolites (MATTILA, HUTTUNEN & AIRAKSINEN unpublished results). Methysergide had as little effect on the gastric secretions as it had on the excretion of 5HT metabolites in the present study. On the other hand, the components of saliva and their probable action on gastric excretions (LEVINE 1965) were not excluded in this study.

An interesting observation is that the carnivorous cat excreted less 5HT metabolites *in toto* and also per ml into the gastric juice than did the herbivorous rabbit and the omnivorous rat. After 5HTP infusion there were less species-specific differences in the excretion of labelled compounds and fewer differences between the various digestive secretions.

The very weak activity of glucuronyltransferase in the cat is well known. Our results indicate that, for some substrates at least, the cat compensates for the lack of glucuronidation by increased formation of sulphate esters. WELCH *et al* (1966) recently reported, however, that cats were unable to form readily not only glucuronide but also O-sulphate of N-acetyl-p-aminophenol *in vivo*. It remains to be seen whether there are different types of sulphate transferase for 5-hydroxyindoles and N-acetyl-p-aminophenol or if some strains of cats have a defect in O-sulphuration of phenolic compounds like Gunn rats have with glucuronidation.

5-Hydroxytryptophol is formed by many mammalian species. It is major metabolite of 5HT in the blood platelets of rabbit (BARTHOLINI *et al* 1964; PAASONEN & AIRAKSINEN 1965). O-glucuronide and O-sulphate of 5-hydroxytryptophol are found in the urine of rats (KVEDER *et al* 1962; AIRAKSINEN 1963). 5-Hydroxytryptophol is a better substrate for glucuronyltransferase than 5HTP, 5HT and 5HIAA (AIRAKSINEN *et al* 1965). The large amount of 5-hydroxytryptophol-O-sulphate in the urine, bile and gastric juice of the cat and the absence of unconjugated 5-hydroxytryptophol indicate that this is also true for the sulphatetransferase. LEMBECK *et al* (1965) recently described only three considerable spots, corresponding to 5HT, 5HIAA and 5HIAA-O-sulphate, in the chromatograms of intestinal mucosa and venous blood of cats after the administration of ^{14}C 5HT into the loop of the small intestine. Since they used only butanol-acetic acid-water solvent,

it is very probable that the last mentioned spot also included 5-hydroxy tryptophol-O-sulphate

For 5HT the "blood-gastrointestinal barrier" seemed to be similar to the blood-brain barrier nonmetabolized 5HT does not penetrate it but its metabolites as well as 5HTP can go through more easily The finding that after ^{14}C 5HTP administration, the portion of 5HT from the total radioactivity excreted was not as high in digestive secretions as in urine also supports the theory that the urinary 5HT is mainly formed in the kidney (AIRAKSINEN & UUSPÄÄ 1961).

Summary

The excretion of radioactive metabolites in urine, bile and gastric, duodenal and intestinal juices were studied in anaesthetized cats and rabbits after intravenous infusion of ^{14}C-labelled 5-hydroxytryptamine (5HT) and 5-hydroxytryptophan (5HTP). The effect of some drugs on the gastric excretion of the metabolites of ^{14}C 5HT was also studied in rats.

About half of the given radioactivity was excreted into the urine within 24 hr both in cats and rabbits After ^{14}C-5HT administration the bile and gastric juice contained more radioactivity than the duodenal and jejunal juices (with pancreatic juice), and the gastric juice of rabbits and rats more than that of cats. In all the gastrointestinal secretions more radioactivity was excreted after ^{14}C 5HTP than after ^{14}C 5HT infusion. 5HT was present in gastrointestinal secretions in the form of the same meta bolites as were found in the urine major metabolites were conjugation products. After d,l 5HTP administration, the unchanged 5HTP (probably d-form) was one of the main components of all fluids studied, the excretions as 5HT itself was also quite high in the urine, other metabolites were similar to those found after 5HT administration. Rats and rabbits excreted 5HT mainly as 5HIAA and glucuronides of 5HT 5-hydroxytryptophol and 5HIAA. In cats the corresponding O-sulphates were major metabolites. Reserpine markedly inhibited the gastric ex cretion of the metabolites of ^{14}C 5HT while methysergide was without any effect. Pheniprazine prevented the inhibitory effect of reserpine, but itself slightly decreased the gastric fluid volume and the total amount of radioactivity in the gastric juice.

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Kristinn Stefánsson

Kristinn Stefánsson died on the 2nd of September 1967 About 1½ years ago a carcinoma of the colon was diagnosed and in July 1966 he underwent a major abdominal operation He recovered rather well after the operation and continued in relatively good health for several months afterwards. At the beginning of this year however it became clear that his condition was rapidly deteriorating. Thus, this previously healthy and active man died shortly before his 64th birthday

Stefánsson was born in 1903 He matriculated in Reykjavík and graduated in 1932 as doctor of medicine from The University of Iceland, Reykjavík. A year later he went abroad and studied pharmacology in Copenhagen, Munich and in London for altogether about 4 years.

At Copenhagen Stefánsson worked in Professor Bock s department in close collaboration with Knud O Moller who later became professor and head of this department and who is known as the author of one of the best student textbooks in pharmacology In Munich, Stefánsson worked with the famous pharmacologist, Professor Straub During these years, several publications appeared by Stefánsson and his co-workers. These publications attest to Stefánsson s great ability as a scientific research worker and emphasize his knowledge of basic pharmacology

Stefánsson was offered a post abroad but he chose to return to settle in his native country On October 1st 1937 he was appointed associate professor in pharmacology at the University at Reykjavík, which at that time was the best chair in pharmacology In 1957 he was finally appointed full professor of the faculty a chair he held until his death. During his life, Stefánsson had lectured in pharmacology for almost 30 years, the only intermission being a year of study in Munich 1938–1939

In small countries like Iceland the same man and particularly if he has great mental ability is often called on to work in more than one field Thus, two years after being appointed associate professor in 1937 Stefánsson in addition to this was given the post of director of The State Import of Drugs and Medicine, which he also held until his death. Due to Stefánsson s great energy and drive The State Import, which is a kind of wholesale firm and pharmacy serving the state-owned hospitals and the dispensing doctors, became greatly expanded and is at present an essential link in the Icelandic health service

Stefánsson sat on many committees, among others, The Icelandic Pharmacopoeia Commission which was established in 1963 and The Icelandic Medical Council which he attended for many years. He was Icelandic delegate to the meetings of The Nordic Pharmacopoeia Council from 1948 until this year During the last 2 years, Stefánsson also attended the meetings of The Nordic Drug Committee, which was established 4 years ago in order to pave the way for further co-operation in the drug field between the Nordic countries. He took a great interest in this work.

During the two last years of his life, Stefánsson sat on the board of editors of this journal. Previously he had been one of the editors of the Icelandic Medical Journal and chairman of The Icelandic Medical Association for 4 years (1957–1961)

Stefánsson was one of the keenest hunters in this country and made many hunting excursions to the central uninhabited highland of Iceland. He also was one of the most enthusiastic anglers in Iceland and caught salmon summer after summer in one of the very best rivers in the country

Professor Kristinn Stefánsson was a lovable and conscientious man. His friends and family mourn his untimely death. He would have been 64 years old to-day

Honoured be his name

Reykjavík, October 8 1967

Torkell Jóhannesson